IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

IN RE: WOLF, Fred R. et al.	
	APPEAL NO. _____
SERIAL NO: 10/606,877	
FOR: METHOD OF IMPROVING ANIMAL TISSUE QUALITY BY SUPPLEMENTING THE ANIMAL DIET WITH MIXED TOCOTRIENOLS	
	BRIEF ON APPEAL
FILED: June 25, 2003	
GROUP ART UNIT: 1615	
CONFIRMATION NO: 5242	

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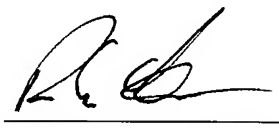

ROBERT A. HODGSON

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I. INTRODUCTION

This is an appeal of the Final Rejection dated April 06, 2007, finally rejecting claims 1, 2, 12-16, 20-21 and 27-29. The appealed claims 1, 2, 12-16, 20-21 and 27-29 are set forth in the attached Claim Appendix.

II. REAL PARTY OF INTEREST

The real parties of interest for this application are Pioneer Hi-Bred International, Inc. and E.I. Du Pont De Nemours and Co., the Assignees of record for this application. The assignments have been recorded at Reel 014240 and Frame 0973 on June 25, 2003; at Reel 014239 and Frame 0702 on June 25, 2003; and at Reel 018324 and Frame 0482 on September 29, 2006.

III. RELATED APPEALS AND INTERFERENCES

None.

IV. STATUS OF CLAIMS

Claims 1-32 were originally submitted June 25, 2003. In a Response to Office Action (Restriction Requirement) dated September 1, 2006, Appellants elected Groups V (claims 12 and 13) and VI (claims 14-16 and 27-29). Rejoinder of Groups V and VI was approved in an Interview Summary mailed August 22, 2006. In an amendment dated January 19, 2007, Appellants canceled claims 3-11, 17-19, 22-26 and 30-32 as drawn to non-elected inventions.

The final rejection mailed April 06, 2007 rejects claims 1, 2, 13, 14-16, 20, 21 and 27-29 as obvious under 35 U.S.C. § 103 over Saunders, et al., U.S. Patent No. 6,977,269 in view of Eenennaam, et al., U.S. Patent Publication No. 2003/0154513; provisionally rejects claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting over claims 1-27 of copending Application No. 11/153,463; and provisionally rejects claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting over claims 1-20 of copending Application No. 11/530,075. The claims here appealed are claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29.

V. STATUS OF AMENDMENTS

An Amendment After Final Rejection was filed on June 5, 2007, following the Examiner's Final Rejection of April 6, 2007. A Notice of Appeal was timely filed on July 2, 2007.

VI. SUMMARY OF CLAIMED SUBJECT MATTER

A. Independent claim 1

Independent claim 1 relates to a method for improving the tissue quality of an animal. (see, e.g., specification pp. 1-2, paragraphs [0017] – [0024]. The method comprises feeding the animal a diet comprising at least 150 ppm mixed tocotrienols. (Specification pp 1-2, paragraph [0018]; Specification, Examples 1-4, pp. 2-8, paragraphs [0025] – [70]).

The mixed tocotrienols may be any mixture which contains at least three of the four known tocotrienols, which are alpha-, beta-, gamma-, and delta-tocotrienol. (Specification p. 2, paragraph [0021]; p. 1, paragraph [0011]). Tissue quality is measured by a number of criteria, including pH, color value, oxidative stability, and purge loss. (Specification p. 3, paragraph [0020]). Furthermore, the diet may comprise a cereal grain crop genetically modified to have elevated mixed tocotrienol levels or oil from plant genetically modified to have elevated mixed tocotrienol levels. (Specification, p. 1, paragraph [0017]).

B. Independent claim 20

Independent claim 20 relates to a method for improving the tissue quality of an animal. See, e.g., specification pp. 1-2, paragraphs [0017] – [0024]. The method comprises feeding the animal a diet comprising 50 ppm to 500 ppm mixed tocotrienols. Specification pp. 1-2, paragraph [0018]; Specification, Examples 1-4, pp. 2-8, paragraphs [0025] – [70].

VII. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1, 2, 13, 14-16, 20, 21 and 27-29 are unpatentable under 35 U.S.C. § 103(a) as being obvious over U.S. Patent No. 6,977,269 to Saunders et al. in view of U.S. Patent Publication No. 2003/0154513 to Eenennaam, et al.

B. Whether claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 are unpatentable as being provisionally rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-27 of copending Application No. 11/153,463.

C. Whether claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 are unpatentable as being provisionally rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-20 of copending Application No. 11/530,075.

VIII. ARGUMENT

A. Claims 1, 2, 13, 14-16, 20, 21 and 27-29 are nonobvious with respect to U.S. Patent No. 6,977,269 to Saunders et al. in view of U.S. Patent Publication No. 2003/0154513 to Eenennaam, et al.

1. Legal Standard for Obviousness

In rejecting claims under 35 U.S.C. § 103, the Examiner bears the initial burden of establishing a prima facie case of obviousness. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992); *see also In re Piasecki*, 745 F.2d 1468, 1472, 223 USPQ 785, 788 (Fed. Cir. 1984). It is incumbent upon the Examiner to establish a factual basis to support the legal conclusion of obviousness. *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006). ("[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). In so doing, the examiner is expected to make the factual determinations set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 17, 148 USPQ 459, 467 (1966). *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1734, 82 USPQ2d 1385, 1391 (2007) ("While the sequence of these questions might be reordered in any particular case, the [*Graham*] factors continue to define the inquiry that controls.")

[T]he scope and content of the prior art ... determined; differences between

> the prior art and the claims at issue are ... ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented.

Id., 127 S.Ct. at 1729-30, 82 USPQ2d at 1338 (quoting *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966)) (internal quotations omitted).

These showings by the examiner are an essential part of complying with the burden of presenting a prima facie case of obviousness. *See Oetiker*, 977 F.2d at 1445, 24 USPQ2d at 1444. Only if this initial burden is met does the burden of coming forward with evidence or argument shift to the appellant. *Id.* at 1445, 24 USPQ2d at 1444. *See also Piasecki*, 745 F.2d at 1472, 223 USPQ at 788. Obviousness is then determined on the basis of the evidence as a whole and the relative persuasiveness of the arguments. *See Oetiker*, 977 F.2d at 1445, 24 USPQ2d at 1444; *Piasecki*, 745 F.2d at 1472, 223 USPQ at 788.

2. Neither Saunders, nor Eenennaam, et al., alone or in combination teach "feeding the animal a diet comprising at least 150 ppm mixed tocotrienols" or "feeding the animal a diet comprising 50 ppm to 500 ppm mixed tocotrienols"

One of the four factual inquiries under *Graham v. John Deere*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966) is ascertaining the differences between the prior art and the claims in issue. The Examiner has erred in this factual inquiry by misinterpreting the prior art references relied upon and thus, the Examiner should be reversed. Neither Saunders et al. or Eenennaam et al. alone or in combination may be properly interpreted as disclosing the step of "feeding the animal a diet comprising at least 150 ppm mixed tocotrienols" in the context

of independent claim 1. Moreover, neither Saunders et al. or Eenennaam et al. alone or in combination may be properly interpreted as disclosing the step of "feeding the animal a diet comprising 50 ppm to 500 ppm mixed tocotrienols" in the context of independent claim 20.

Independent claims 1 and 20 both require "mixed tocotrienols". "Mixed tocotrienols" is defined in the specification as "any mixture that contains at least three of the four known tocotrienols" and "any mixture of tocotrienols comprising significant quantities of at least three of the four known tocotrienols". Specification, p. 1, paragraphs [0018], [0021]. Neither Saunders et al. or Eenennam et al. teach improving the tissue quality of an animal comprising feeding the animal a diet of mixed tocotrienols.

The Examiner cites to col. 3, ll. 18-21 of Saunders as teaching a method of improving the tissue quality of an animal by feeding the animal Vitamin E. Citing Eenennaam et al., the Examiner states that Vitamin E describes a broad category of compounds, including the four tocotrienols. (Office Action of April 6, 2007, p. 2). Saunders et al. does not teach improving the tissue quality of an animal by feeding an animal a diet including mixed tocotrienols. Instead, Saunders et al. teaches a method of improving the tissue quality of an animal by feeding the animal a diet including tocopherols, namely alpha- and gamma-tocopherol. *See, e.g.,* Saunders et al., col. 1, ll. 41-51 (noting that prior research has indicated that "supplementation of vitamin E in the form of alpha-tocopherol acetate at supernutritional levels is an effective means for improving meat quality") (emphasis added); Saunders et al., col. 3, ll. 18-34) (stating that the present invention taught in Saunders et al. "is a method for improving the tissue quality of an animal, comprising feeding the a diet including gamma-

tocopherol in an amount effective to improve the tissue quality") (emphasis added). Saunders et al. thus does not teach, suggest, or disclose a method for improving animal tissue quality by feeding the animal a diet comprising mixed tocotrienols.

Further, the terms "Vitamin E", "tocopherol" and "tocotrienol" are not analogous terms. As taught by Eenennaam et al., "[a]lthough α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols are sometimes referred to collectively as 'vitamin E', vitamin E is more appropriately defined chemically as α tocopherol." Eenennaam et al., p. 1, paragraph [0004]. The specification further teaches prior research has taught that supplementation of Vitamin E in the form of alpha-tocopherol acetate is an effective means for improving meat quality. Specification, p. 1, paragraph [0008]. However, the specification distinguishes between alpha-tocopherol and the present invention, stating that "ATA [alpha-tocopherol acetate] does not, however, provide consistent results, and must be incorporated into the animal diet over a fairly long period of time, depending on the dietary concentration. Supernautritional levels of ATA are sometimes used commercially in cattle feed, though not yet for poultry or swine, owing to an inadequate cost-to-benefit ratio." Specification, p. 1, paragraph [0009].

It is clear from the specification that tocopherols and tocotrienols are different and distinct compounds. Tocotrienols, while being lesser-known compounds and which appear less frequently in nature than tocopherols, have been shown by certain studies to exhibit greater anti-oxidative activity than tocopherols. Specification, p. 1, paragraphs [0010] – [0011] (noting that in vitro studies have shown that alpha- and gamma- tocotrienols have greater anti-oxidative activity than alpha-tocotrienol acetate).

Moreover, the state of the art at the time the application was filed clearly distinguished between the tocopherols and the tocotrienols. The art describes tocopherols and tocotrienols as being distinct compounds, both structurally and functionally. See Yap et al., which states that:

> [t]he difference between the tocopherols and tocotrienols lies mainly in the former having a saturated phytyl chain, while that of the latter is unsaturated, with three double bonds at 3', 7' and 11' positions . . . In recent years, tocotrienols have generated much interest as they have been reported to posses certain biological activities that were not observed with the tocopherols, including cholesterol-lowering activity . . . anticancer and tumor-suppressing activities . . . antioxidant properties . . . and anti-aggregation of blood platelets.

Yap et al., p. 53 (Exhibit A). U.S. Patent 7,154,029 further distinguishes tocopherols from tocotrienols, noting that the compounds differ structurally due to the presence of "three double bonds in the hydrocarbon side chain of tocotrienols" and that this difference is due to the fact that "[t]ocopherol side chains are derived from phytyl-pyrophosphate (PP), and the tocotrienol side chains are believed to be derived from geranylgeranyl-PP ". U.S. Patent 7,154,029, col. 1 (Exhibit B).

Eenennaam et al. additionally does not teach a method of improving the tissue quality of an animal comprising feeding the animal a diet of mixed tocotrienols. Nowhere in Eenennaam et al. is there taught an animal diet or transgenic plant which comprises mixed tocotrienols. Contrary to the Examiner's suggestion, Eenennaam et al. does not present any evidence or data that the transgenic plants disclosed therein contain elevated levels of mixed tocotrienols. Eenennaam et al. discloses data showing elevated levels of tocopherols in the disclosed transgenic plants, but no data or teaching of transgenic plants with elevated levels

of mixed tocotrienols. *See, e.g.* Eenennaam et al., Table 5, pp. 33-34 (disclosing plants with altered levels of gamma-tocopherol); Example 4, pp. 36-37; FIG. 34 (comparing the gamma-tocopherol and gamma-tocotrienol levels in plants); Example 7, pp. 39-40 (discussing the tocopherol levels of transformed plants); Examples 8-9 (disclosing the measurement of alpha-, gamma-, and delta-tocopherol levels in transformed plants).

Accordingly, neither Saunders et al. or Eenennaam et al., either alone or in combination, teach a method of improving the tissue quality of an animal, comprising feeding the animal a diet of mixed tocotrienols. In view of the differences between the claimed invention and the references of record, the claimed invention is not rendered obvious by Saunders et al. in view of Eenennaam et al... Therefore, the § 103(a) rejection of claims 1, 2, 13, 14-16, 20, 21 and 27-29 with respect to Saunders et al. in view of Eenennaam, et al. should be reversed.

B. Claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 are patentable over claims 1-27 of copending Application No. 11/153,463

The Examiner has erred in provisionally rejecting claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims1-27 of copending Application No. 11/153,463 ("the '463 application"). The '463 application does not teach all of the elements of the present invention. Appellants' independent claims 1 and 20 recite a method of improving the tissue quality of an animal, comprising feeding the animal a diet comprising mixed tocotrienols.

In contrast, the '463 application teaches and claims a method of improving the tissue quality of an animal comprising feeding the animal a diet comprising oleic acid and selected tocols. The "selected tocols" are defined in the '463 application as "one or more of the tocotrienols (TT), gamma-tocopherol (GT) or a mixture of at least one tocotrienol and gamma-tocopherol." '463 Application, p. 1, paragraph [0009]. The '463 application does not teach or suggest a method of improving the tissue quality of an animal comprising feeding the animal a diet of mixed tocotrienols, as required by Appellants' independent claims 1 and 20. As discussed *supra*, "mixed tocotrienols" is defined in the specification as "any mixture that contains at least three of the four known tocotrienols". Specification, p. 2, paragraph [18] (emphasis added). Further, the '463 application does not teach or claim an animal diet using mixed tocotrienols alone.

The '463 application accordingly does not teach all of the limitations of independent claims 1 and 20. Therefore the provisional rejection of claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims1-27 of the '463 application should be reversed.

C. Claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 are patentable over claims 1-20 of copending Application No. 11/530,075

The Examiner has erred in provisionally rejecting claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims1-20 of copending Application No. 11/530,075 ("the '075 application"). The '075 application does not teach all of the elements of the present

invention. Appellants' independent claims 1 and 20 recite a method of improving the tissue quality of an animal, comprising feeding the animal a diet comprising mixed tocotrienols.

In contrast, the '075 application teaches and claims a method of improving the meat quality of an animal comprising feeding the animal a diet comprising oleic acid, tocols and a non-tocol anti-oxidant. The '075 application states that the dietary tocols "may be one or more of the tocopherols or tocotrienols or a mixture of tocopherols and/or tocotrienols. The tocotrienols may be a mixture of two or more of the four known tocotrienols or a single tocotrienol." '075 Application, p. 1, paragraph [0016]. The '075 application does not teach or suggest a method of improving the tissue quality of an animal comprising feeding the animal a diet of mixed tocotrienols, as required by Appellants' independent claims 1 and 20. As discussed *supra*, "mixed tocotrienols" is defined in the specification as "any mixture that contains at least three of the four known tocotrienols". Specification, p. 2, paragraph [18] (emphasis added). Further, the '075 application does not teach or claim an animal diet using mixed tocotrienols alone.

The '075 application accordingly does not teach all of the limitations of independent claims 1 and 20. Therefore the provisional rejection of claims 1, 2, 12, 13, 14-16, 20, 21 and 27-29 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of the '075 application should be reversed.

IX. CONCLUSION

For the above-stated reasons, it is submitted that the claims are in a condition for allowability. The decision of the Examiner, therefore, should be reversed and the case allowed.

Enclosed herein please find the Appeal Brief. Please charge Deposit Account No. 26-0084 the amount of $500.00 for this Appeal Brief. No other fees or extensions of time are believed to be due in connection with this appeal; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Respectfully submitted,

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- RAH/bjh -

X. APPENDIX - CLAIMS

1. A method of improving the tissue quality of an animal, comprising feeding the animal a diet comprising at least 150 ppm mixed tocotrienols.

2. The method of claim 2 wherein the tissue is meat and the quality of the meat is measured by criteria selected from the group consisting of pH, improved color value, improved oxidative stability, and reduced purge.

12. The method of claim 1 wherein the animal is poultry.

13. The method of claim 12 wherein the animal is cattle.

14. The method of claim 1 wherein the diet comprising at least 150 ppm mixed tocotrienols comprises a cereal grain crop genetically modified to have elevated mixed tocotrienol levels.

15. The method of claim 14 wherein the cereal grin crop is corn.

16. The method of claim 1 wherein the diet comprising at least 150 ppm mixed tocotrienols comprises oil from a plant that has been genetically modified to have elevated mixed tocotrienol levels.

20. A method of improving the tissue quality of an animal, comprising feeding the animal a diet comprising 50 ppm to 500 ppm mixed tocotrienols.

21. The method of claim 20 wherein the tissue is meat and the quality of the meat is measured by criteria selected from the group consisting of increased pH, improved color value, improved oxidative stability and reduced purge.

27. The method of claim 20 wherein the diet comprising 50 ppm to 500 ppm mixed tocotrienols comprises a cereal gain crop genetically modified to have elevated mixed tocotrienol levels.

28. The method of claim 27 wherein the cereal grain crop is corn.

29. The method of claim 20 wherein the diet comprising 50 ppm to 500 ppm mixed tocotrienols comprises an oil from a plant that has been genetically modified to have elevated mixed tocotrienol levels.

XI. EVIDENCE APPENDIX

Only evidence of record has been relied upon in this appeal.

Exhibit A: Yap, Siew Ping et al.,(2003) Influence of route in administration on the absorption and disposition of α-, γ-, and δ-tocotrienols in rats in *Journal of Pharmacy and Pharmacology*, Vol. 55: 53-58

First cited by Applicants in June 5, 2007 Amendment After Final, p. 3.

Exhibit B: U.S. Patent No. 7,154,029

First cited by Applicants in June 5, 2007 Amendment After Final, pp. 3-4.

XII. RELATED PROCEEDING APPENDIX

None

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Influence of route of administration on the absorption and disposition of α-, γ- and δ-tocotrienols in rats

Siew Ping Yap, Kah Hay Yuen and Ai Beoy Lim

Abstract

A study was conducted to evaluate the bioavailability of α-, γ- and δ-tocotrienols administered via oral, intravenous, intramuscular and intraperitoneal routes in rats. Three separate experiments, each conducted according to a two-way crossover design, were carried out to compare intravenous and oral, intramuscular and oral, and intraperitoneal and oral administration. Oral absorption of all three tocotrienols was found to be incomplete. Of the three tocotrienols, α-tocotrienol had the highest oral bioavailability, at about 27.7±9.2%, compared with γ- and δ-tocotrienols, which had values of 9.1±2.4% and 8.5±3.5%, respectively. Such biodiscrimination was also observed in their total clearance rates (estimated from the intravenous data). α-Tocotrienol showed the lowest clearance rate at about 0.16 L kg^{-1} h^{-1}, whereas that of δ- and γ-tocotrienols was quite similar, with values of 0.24 and 0.23 L kg^{-1} h^{-1}, respectively. Interestingly, all three tocotrienols were found to be negligibly absorbed when administered intraperitoneally and intramuscularly. Thus, these two routes of administration should be avoided when evaluating the biological activities of the tocotrienols in whole animal experiments.

Introduction

Vitamin E comprises eight compounds, namely four tocopherols and four tocotrienols, which share similar structural features of a chromanol head and a 16-carbon phytyl chain. Both tocopherols and tocotrienols are designated as α, β, γ and δ, depending on the number and positions of methyl groups on the chromanol ring. The difference between the tocopherols and tocotrienols lies mainly in the former having a saturated phytyl chain, while that of the latter is unsaturated, with three double bonds at 3′, 7′ and 11′ positions (Kamal-Eldin & Appelqvist 1996).

In recent years, tocotrienols have generated much interest as they have been reported to posses certain biological activities that were not observed with the tocopherols, including cholesterol-lowering activity (Qureshi et al 1991, 1995), anticancer and tumour-suppressing activities (Goh et al 1994; Nesaretnam et al 1998), antioxidant properties (Serbinova et al 1992; Kooyenga et al 1997) and anti-aggregation of blood platelets (Mahadevappa et al 1991). Despite the growing interest, there is a paucity of information with regard to their bioavailability and absorption, especially via different routes of administration. We have previously shown that the oral bioavailability of the tocotrienols determined from human studies was markedly increased when taken with food, and their biological half-lives were relatively short, being almost 4- to 5-fold shorter compared with that of α-tocopherol (Yap et al 2001). However, such information is lacking in other animal species even though animals such as rats and guinea-pigs have been used in evaluating the biological activities of the tocotrienols. The present study was conducted to determine the pharmacokinetics and bioavailability of α-, γ- and δ-tocotrienols given via different routes of administration in rats, namely via the oral, intravenous, intramuscular and intraperitoneal routes. In additional, their oral bioavailability was estimated with reference to the intravenous route of administration.

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Acknowledgement: We wish to thank Carotech Pte Ltd for the generous supply of Tocomin 50%.

EXHIBIT A

Materials and Methods

Materials

Tocomin 50%, containing 21.7%, 5.9%, 11.8% and 11.1% of γ-, δ-, α-tocotrienol and α-tocopherol respectively, was obtained from Carotech Pte Ltd (Ipoh, Malaysia). The rest of the Tocomin 50% consisted mainly of palm olein, plant squalene and phyto-sterol complex, with trace amounts of phyto-carotenoid complex and co-enzyme Q10. Tocotrienol standard kit was purchased from Merck (Darmstadt, Germany). Labrasol and Tween 80 were purchased from Gattefossé (Cedex, France) and Sigma (St Louis, MO, USA), respectively. Soybean oil was purchased from Yee Lee Edible Oils Pte Ltd (Ipoh, Malaysia). All other solvents used were either of analytical reagent grade or HPLC grade and were purchased from either Merck or Ajax Chemicals (Auburn, Australia).

Animals

Male Sprague–Dawley rats were obtained from the animal holding of the University of Science Malaysia.

Preparation

For oral, intramuscular and intraperitoneal administration, Tocomin 50% was diluted with soybean oil to give α-, γ- and δ-tocotrienol concentrations of approximately 11.8 mg g^{-1}, 21.7 mg g^{-1} and 5.9 mg g^{-1}, respectively (5 mg mixed tocotrienols). For intravenous administration, Tocomin 50% was emulsified in water using 2% Tween 80 and 14% Labrasol. The emulsion was homogenized at 13 500 rev min^{-1} for 10 min and filtered through a 0.45-μm filter. The first 1 mL of the filtrate was discarded. The mean droplet size of the emulsion, designated the volume median diameter, was determined by a laser diffraction technique using a Malvern Mastersizer S diffraction particle analyser (Malvern Instruments, Worcestershire, UK) and was found to be 0.55 ± 0.05 μm. The final emulsion contained equivalent concentrations of α-, γ- and δ-tocotrienols as the oily solution. The concentration of the tocotrienols in both the oily preparation and the emulsion after filtration was assayed using the HPLC method reported by Yap et al (1999). The volume of both preparations was adjusted to give the desired dose of 5 mg mixed tocotrienols, being approximately 135 μL in volume.

In-vivo absorption studies

The study was approved by the Ethics Committee on Animal Studies, University of Science Malaysia. Three separate experiments were conducted to study the relative absorption of the tocotrienols via different routes of administration; the first was between the intravenous and oral routes, the second was between the intramuscular and oral routes, and the third was between the intraperitoneal and oral routes. Each experiment was conducted using six adult male Sprague–Dawley rats according to a two-way crossover study design with a washout period of 1 week. For each experiment, the rats were randomly divided into two groups of three rats each and administered the tocotrienols according to the sequence shown in Table 1.

Table 1 Sequence of administration following a crossover design.

Study	Group	Sequence of administration	
		1st week	2nd week
Oral versus intravenous	I	Oral	Intravenous
	II	Intravenous	Oral
Oral versus intramuscular	I	Oral	Intramuscular
	II	Intramuscular	Oral
Oral versus intraperitoneal	I	Oral	Intraperitoneal
	II	Intraperitoneal	Oral

All the animals were fasted for 12 h before drug administration and also during the study period, but were allowed free access to water throughout the experiment. For all routes of administration, the dose used was 5 mg of mixed tocotrienols (approx. 1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively). The preparation was given orally via oral intubation. In the case of intraperitoneal and intramuscular administrations, the preparations were injected into the peritoneal cavity and the thigh muscle, respectively, using a 27G1/2 needle. For intravenous administration, the emulsion was injected into the tail vein. The animals were then placed in restraining cages and blood samples (approx. 0.5 mL) were collected from the tail vein into heparinized tubes at 0 (before administration), 1, 2, 3, 4, 6, 8, 10, 14 and 22 h after administration. In the case of intravenous administration, two additional blood samples were collected at 15 and 30 min after dosing. The blood samples were then centrifuged for 10 min at 12 800 *g*, and the plasma transferred into new Eppendorf tubes for storage at -20°C until analysis.

Analysis of α-, γ- and δ-tocotrienols

Plasma concentrations of α-, γ- and δ-tocotrienols were determined using a high-performance liquid chromatography (HPLC) method reported previously (Yap et al 1999).

Data analysis

The bioavailability of the tocotrienols after the different routes of administration was assessed using the pharmacokinetic parameters, peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}) and total area under the plasma concentration–time curve ($AUC_{0-\infty}$), which were estimated from the plasma concentration–time data. Both C_{max} and t_{max} were obtained directly from the plasma concentration values (Weiner 1981), while the $AUC_{0-\infty}$ was calculated by adding the area from time zero to the last detectable sampling time t (AUC_{0-t}) and the area from time t to infinity ($AUC_{t-\infty}$). The former was calculated using the trapezoidal formula and the latter by dividing the last measurable plasma drug concentration with the elimination rate constant (k_e). In all cases, the $AUC_{t-\infty}$ was

found to be less than 20% of the $AUC_{0-\infty}$. The k_e was estimated from the terminal slope of the individual plasma concentration–time curves after logarithmic transformation of the plasma concentration and application of linear regression (Gibaldi & Perrier 1982). From the intravenous data, the apparent volume of distribution (Vd) of the three tocotrienols was calculated as dose/($AUC_{0-\infty} \times k_e$), and the total clearance (CL_{tot}) was calculated using the relationship $k_e \times Vd$. In the experiment comparing the oral and intravenous routes, only the extent of bioavailability was compared using the parameter $AUC_{0-\infty}$. The absolute bioavailability was estimated by dividing the $AUC_{0-\infty}$ obtained from the oral administration with that obtained from intravenous administration.

Statistical analysis

The parameter values of both $AUC_{0-\infty}$ and k_e, obtained from the study comparing oral and intravenous administrations, were analysed using an analysis of variance procedure appropriate for a crossover study design. However, in the comparison of the CL_{tot} values of the three tocotrienols, analysis was performed using an analysis of variance procedure appropriate for a randomized block study design, followed by Tukey's test for pairwise comparison when a statistically significant difference was observed. For the parameter t_{max}, the values obtained for the three tocotrienols were analysed using the Friedman's test. A statistically significant difference was considered at $P < 0.05$.

Results

Figure 1A shows the plasma profiles of the tocotrienols obtained by comparing intravenous and oral administration. It can be seen from the figure that the plasma profiles of the three tocotrienols after intravenous administration appeared to be biphasic in nature, being characterized by an initial rapid decline for about 2 h after dosing, followed by a more gradual decline after this phase. This is clearly demonstrated by plotting the intravenous data on a $\log_{10}$ concentration–time scale, as shown in Figure 1B, suggesting that the pharmacokinetics of the tocotrienols might be better fitted or described by a two-compartment pharmacokinetic model. The initial rapid decline in plasma concentration could be due to redistribution of the compounds from the plasma compartment to other tissues and organ systems. In the case of oral dosing, the plasma concentrations of the tocotrienols showed a rapid increase, reaching a peak at approximately 3 h after dosing, and a gradual decline thereafter, being typical of profiles obtained with extravascular administration. The mean values of the pharmacokinetic parameters of C_{max}, $AUC_{0-\infty}$, t_{max} and k_e obtained from the individual plasma profiles after intravenous and oral dosing are given in Table 2. Of the three compounds, α-tocotrienol achieved the highest mean C_{max} value after oral dosing, followed by γ- and δ-tocotrienols. This was also reflected in the mean $AUC_{0-\infty}$ values; α-tocotrienol had the highest value, while δ-tocotrienol had the lowest. However, the administered doses of the three

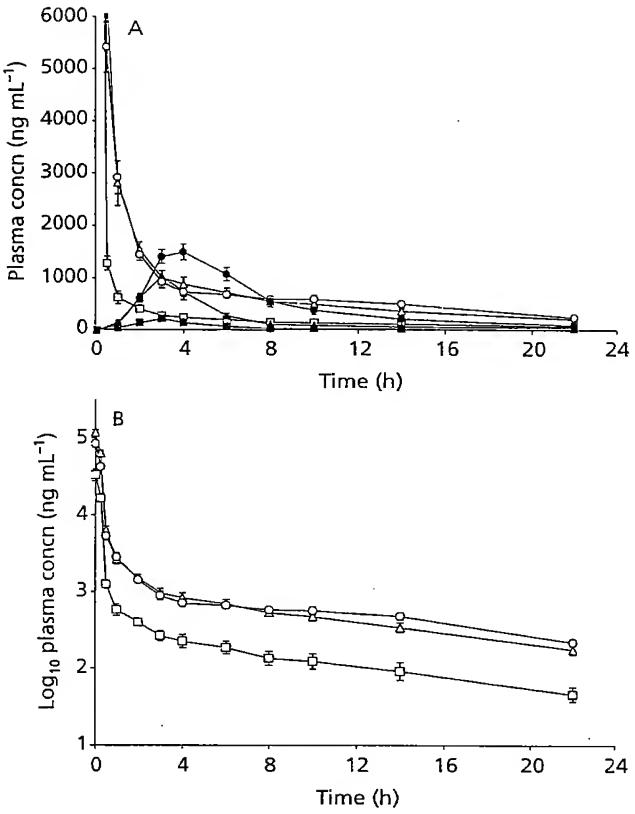

Figure 1 A. Plasma concentration–time profiles (mean ± s.e.m., n = 6) of α-, γ- and δ-tocotrienols after intravenous and oral administration of a single dose of 5 mg mixed tocotrienols (approx. 1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively). ○, intravenous α-tocotrienol; ●, oral α-tocotrienol; △, intravenous γ-tocotrienol; ▲, oral γ-tocotrienol; □, intravenous δ-tocotrienol; ■, oral δ-tocotrienol. B. Log_{10} plasma concentration–time profiles (mean ± s.e.m., n = 6) of α-, γ- and δ-tocotrienols after intravenous administration of a single dose of 5 mg mixed tocotrienols (approx. 1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively). ○, α-tocotrienol; △, γ-tocotrienol; □, δ-tocotrienol.

compounds were not in the same rank order. While the dose of α-tocotrienol was almost 2-fold lower than that of γ-tocotrienol, its mean C_{max} and $AUC_{0-\infty}$ values were markedly higher than those of the latter, suggesting that the oral bioavailability and/or disposition of the two compounds was different.

Referring to Table 2, it can be seen that the $AUC_{0-\infty}$ values obtained from intravenous dosing were significantly higher than those obtained from oral dosing ($P < 0.01$), suggesting that oral absorption was not complete. As shown in Table 2, α-tocotrienol had an estimated absolute bioavailability of approximately 27.7%, whereas the absolute bioavailability of γ-tocotrienol was only about 9.1%. In the case of δ-tocotrienol, its estimated absolute bioavailability was also quite low, being similar to that of γ-tocotrienol, with a value of only about 8.5%.

Notwithstanding the above discrepancy, however, the t_{max} values of all three compounds were quite comparable

Table 2 Pharmacokinetic parameters of α-, γ- and δ-tocotrienols after oral and intravenous administration of 5 mg mixed tocotrienols (1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively).

Parameter	α-Tocotrienol		γ-Tocotrienol		δ-Tocotrienol	
	Oral	Intravenous	Oral	Intravenous	Oral	Intravenous
C_{max} (ng mL^{-1})	1614.4±347.4	–	1023.2±262.3	–	223.1±80.4	–
t_{max} (h)	3.3±0.5	–	3.0±0.0	–	2.8±0.4	–
Vd (L kg^{-1})	–	1.68±0.28	–	2.64±1.05	–	2.77±1.14
k_e (h^{-1})	0.128±0.043	0.093±0.009	0.125±0.050	0.098±0.032	0.139±0.068	0.094±0.026
$AUC_{0-\infty}$ (h ng mL^{-1})	10580.0±2741.5	39254.4±5880.6*	4360.7±1083.9	48216.1±5312.1*	1038.9±269.4	13188.7±3773.7*
CL_{tot} (L kg^{-1} h^{-1})	–	0.155±0.021†	–	0.231±0.026	–	0.242±0.064
Bioavailability (%)	27.7±9.2		9.1±2.4		8.5±3.5	

*$P < 0.01$, significantly different compared with oral dosing. †$P < 0.01$, significantly different compared with δ- and γ-tocotrienols.

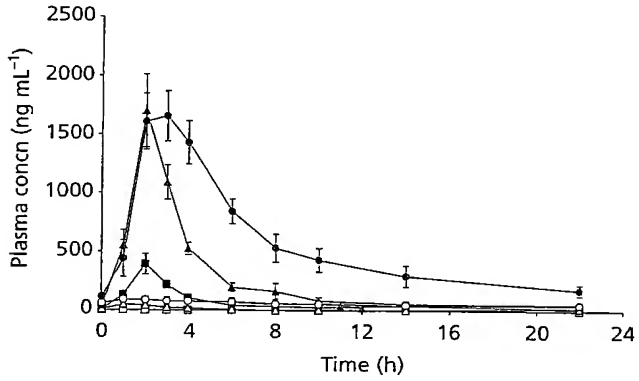

Figure 2 Plasma concentration–time profiles (mean±s.e.m., n = 6) of α-, γ- and δ-tocotrienols after intramuscular and oral administration of a single dose of 5 mg mixed tocotrienols (approx. 1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively). ○, intramuscular α-tocotrienol; ●, oral α-tocotrienol; △, intramuscular γ-tocotrienol; ▲, oral γ-tocotrienol; □, intramuscular δ-tocotrienol; ■, oral δ-tocotrienol.

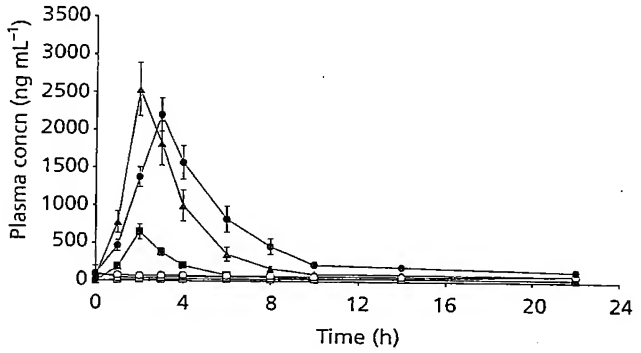

Figure 3 Plasma concentration–time profiles (mean±s.e.m., n = 6) of α-, γ- and δ-tocotrienols after intraperitoneal and oral administration of a single dose of 5 mg mixed tocotrienols (approx. 1.50, 2.75 and 0.75 mg of α-, γ- and δ-tocotrienol, respectively). ○, intraperitoneal α-tocotrienol; ●, oral α-tocotrienol; △, intraperitoneal γ-tocotrienol; ▲, oral γ-tocotrienol; □, intraperitoneal δ-tocotrienol; ■, oral δ-tocotrienol.

($P > 0.05$), with a mean value of about 3 h, indicating that their rate of absorption was similar. It can also be noted from Table 2 that the estimated elimination rate constant of the three tocotrienols was quite similar. Moreover, for all three compounds, the k_e values estimated from the oral administration data were similar to those estimated from the intravenous administration data ($P > 0.05$). It is interesting to note that the apparent Vd of the three compounds (calculated from the intravenous data) was also different. While both γ- and δ-tocotrienols had quite similar Vd values of about 3.0 L kg^{-1}, that of α-tocotrienol tended to be smaller, with a value of about 1.7 L kg^{-1}. Multiplying the Vd values with the respective k_e values, showed that δ- and γ-tocotrienols had similar CL values of about 0.24 L kg^{-1} h^{-1} and 0.23 L kg^{-1} h^{-1}, respectively, whereas that of α-tocotrienol was significantly smaller ($P < 0.01$), with a value of 0.16 L kg^{-1} h^{-1}.

Figure 2 shows the plasma concentrations of the tocotrienols obtained by comparing intramuscular and oral administration. It can be seen from the figure that the tocotrienols were negligibly absorbed when given intramuscularly as evidenced by the low plasma concentrations obtained, which were essentially comparable with the baseline values measured before administration of the compounds. In the case of oral administration, the plasma concentration profiles of all three compounds were similar to the profiles obtained orally in the previous experiment comparing intravenous and oral administration.

The plasma concentration profiles obtained by comparing intraperitoneal and oral administration are shown in Figure 3. As for intramuscular administration, the tocotrienols were found to be negligibly absorbed when given intraperitoneally; the plasma concentrations obtained were essentially comparable with the baseline concentrations. On the other hand, the plasma concentrations obtained after oral administration appeared to be slightly higher than those in the two previous experiments. This discrepancy may be attributed to the smaller bodyweight of the rats used in this experiment compared with the previous

experiments. The mean weight of the rats used in the intraperitoneal versus oral study was 221 ± 18.5 g, whereas the mean bodyweight of the rats used in the first and second study was higher, being 280.0 ± 22.9 g and 277.3 ± 26.4 g, respectively.

Discussion

Differences among the three tocotrienols lie mainly in the number of methyl groups in the chromanol ring of the molecules. α-Tocotrienol has three methyl groups, γ-tocotrienol has two and δ-tocotrienol has one. It appears that such differences also lead to differences in their bioavailability and disposition. α-Tocotrienol not only showed the highest oral bioavailability compared with the other two, but also had the lowest apparent Vd and CL rate. Similar preferential absorption of α-tocotrienol over δ- and γ-tocotrienols has also been observed by Qureshi et al (1991) in pigs. In our previous study (Yap et al 2001), conducted to evaluate the oral bioavailability of the tocotrienols under different food status using human volunteers, the observed plasma concentrations as well as the $AUC_{0-\infty}$ values of α-tocotrienol were similar to those of γ-tocotrienol, even though the dose of the former was approximately half that of the latter. Moreover, from studies using lymphatic cannulated rats, Ikeda et al (1996) demonstrated that α-tocotrienol was preferentially absorbed compared with γ- and δ-tocotrienols, which may explain its higher oral bioavailability over the other two compounds observed in our study. They suggested that the mechanism of preferential absorption could be due to differences in their micellar solubility, affinity for intestinal brush border membranes, transport in enterocytes or their incorporation into chylomicrons or a combination of these processes, which in turn might be related to differences in the lipophilicity of the molecules. Since α-tocotrienol has three methyl groups compared with two in γ-tocotrienol and one in δ-tocotrienol, the lipophilicity of the three molecules would be expected to be different, with α-tocotrienol having the highest lipophilicity, followed by γ- and δ-tocotrienols. This was reflected in the elution time of the three compounds during analysis using reversed phase chromatography, where the elution is influenced by the lipophilicity of the molecules. The δ-tocotrienol, being least lipophilic, has the fastest elution time, followed by γ- and α-tocotrienols. Since the lipophilicity of a molecule can affect its passage across biological membranes and transport into the lymphatic system, the higher bioavailability obtained with α-tocotrienol might in part be related to its higher lipophilicity compared with γ- and δ-tocotrienols.

Such biodiscrimination has also been reported with the tocopherols. For example, plasma and tissue concentrations of α-tocopherol were observed to be 2–3 times higher than those of γ-tocopherol, even though the diet of the subjects studied contained more γ than α-tocopherol (Bieri & Evarts 1973). This discrepancy was attributed to the presence of α-tocopherol transfer protein, which has high selectivity in regulating the secretion of α-tocopherol from the liver as well as in maintaining its plasma concentrations (Kayden & Traber 1993). Hosomi et al (1997) demonstrated that the methyl group at position 5 of the chromanol ring, which is found in α-tocopherol (and also in α-tocotrienol), was important for recognition by this regulatory protein. However, it is not known if the α-tocopherol transfer protein has similar effects on the disposition of α-tocotrienol in-vivo. Ikeda et al (1996) have also suggested that a carrier protein specific for α-tocotrienol might be present in the intestinal cells.

From the intramuscular and intraperitoneal studies, it was found that the tocotrienols were essentially negligibly absorbed when given as an oily injection via these two routes of administration. Negligible absorption was similarly observed when the intramuscular administration was repeated using the tocotrienol emulsion (instead of an oily injection) that was utilized in the earlier intravenous study (data not shown). The negligible absorption via these two routes could be owing to a lack of partitioning of the compounds out of the lipidic vehicle for absorption in the peritoneal compartment or muscle. It is known that intraluminal processing of orally administered lipophilic drugs contained in a lipidic vehicle, which results in the formation of mixed micelles with bile salts, is essential for their absorption (MacGregor et al 1997). Such processing is absent in intramuscular and intraperitoneal administrations, which may explain the negligible absorption of the tocotrienols administered via these two routes. Therefore, these two modes of dosing should be avoided when conducting studies to evaluate the biological activities of the tocotrienols in whole animal experiments. In a study conducted to evaluate the effects of α-tocopherol and tocotrienols on HMG-CoA reductase activity in hamsters, in which the compounds were given intraperitoneally, Khor & Ng (1999) reported that α-tocopherol, but not the tocotrienols, could be determined in the serum and liver. This could be due to negligible absorption of the tocotrienols when given via this route. In another study in which the tocotrienols were also given intraperitoneally to hamsters, Khor et al (2000) reported that the inhibitory effect of the tocotrienols on the HMG-CoA reductase activity tended to decline with increase in the dose level. This somewhat inverse activity versus dose relationship might be an artefact of the study, as it was shown in our study that the tocotrienols were essentially not absorbed when given via the intraperitoneal route.

In conclusion, absorption of tocotrienols was found to be low and incomplete via the oral route. There appeared to be biodiscrimination in the absorption and disposition among the three homologues. In addition, they were found to be negligibly absorbed when administered intraperitoneally and intramuscularly.

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(12) **United States Patent**
Cahoon et al.

(10) **Patent No.: US 7,154,029 B2**
(45) **Date of Patent: Dec. 26, 2006**

(54) **COMPOSITIONS AND METHODS FOR ALTERING TOCOTRIENOL CONTENT**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 568 days.

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(51) **Int. Cl.**

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C12N 15/82	(2006.01)
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(52) **U.S. Cl.** **800/320**; 800/287; 800/298; 800/306; 800/312; 800/317.2; 800/322; 536/23.1; 536/23.2; 536/23.6; 435/320.1; 435/419

(58) **Field of Classification Search** 800/306, 800/312, 317.2, 322, 278, 285, 286, 287, 800/298, 320, 320.1, 320.2, 320.3; 536/23.1, 536/23.2, 23.6; 435/320.1, 419
See application file for complete search history.

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(57) **ABSTRACT**

The invention provides isolated nucleic acids and their encoded polypeptides that alter tocol content in seeds. The invention further provides expression cassettes, host cells and transformed plants containing the nucleic acids. The present invention further provides methods for altering tocol content in seeds.

37 Claims, No Drawings

EXHIBIT B

COMPOSITIONS AND METHODS FOR ALTERING TOCOTRIENOL CONTENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. application Ser. No. 60/366,757 filed Mar. 22, 2002, which is herein incorporated in its entirety by reference.

FIELD OF THE INVENTION

The present invention relates to the field of plant molecular biology, particularly to the isolation of genes. The invention further relates to the use of the genes to host cells. More specifically, this invention pertains to nucleic acid fragments encoding homogentisate geranylgeranyl transferase in plants.

BACKGROUND OF THE INVENTION

Tocotrienols are vitamin E-related compounds whose occurrence in plants is limited primarily to the seeds and fruits of most monocot species (e.g., palm, wheat, rice and barley). Tocotrienols are structurally similar to tocopherols, including α-tocopherol or vitamin E, which occur ubiquitously in the plant kingdom as well as in photosynthetic microbes such as *Synechocystis*. Tocotrienols and tocopherols both contain a chromanol head group that is linked to a hydrocarbon side chain. The only structural difference between these molecules is the presence of three double bonds in the hydrocarbon side chain of tocotrienols. This difference is related to the biosynthetic origins of the side chains. Tocopherol side chains are derived from phytylpyrophosphate (PP), and the tocotrienol side chains are believed to be derived from geranylgeranyl-PP (Soll, J. et al. (1980) *Arch. Biochem. Biophys.* 204:544–550).

Four forms or molecular species of tocopherols and tocotrienols occur in nature: α, β, γ and δ. These molecular species contain different numbers of methyl groups that are bound to the aromatic portion of the chromanol head. Like tocopherols, tocotrienols are potent lipid-soluble antioxidants and therefore have considerable nutritive value in human and animal diets (Packer, L. et al. (2001) *J. Nutr.* 131:369S–373S). In addition, tocotrienols are believed to have therapeutic properties including a demonstrated ability to down regulate cholesterol biosynthesis (Theriault, A. et al. (1999) *Clin. Biochem.* 32:309–319; Qureshii, A. A. et al. (1986) *J. Biol. Chem.* 261:10544–10550).

It has been speculated that the first committed step in the biosynthesis of tocotrienols involves the condensation of geranylgeranyl-PP and homogentisate to form 2-methyl-6-geranylgeranylbenzoquinol (Soll, J. et al. (1980) *Arch. Biochem. Biophys.* 204:544–550). The enzyme that catalyzes this reaction can thus be functionally described as a homogentisate geranylgeranyl transferase (HGGT).

Functional identification of genes or cDNAs encoding HGGT polypeptides has yet to be reported. The lack of these nucleic acids limits efforts to manipulate the biosynthesis of the nutritionally important tocotrienols in plants and microbial hosts. The problem to be solved, therefore, is in identifying the nucleic acids that encode polypeptides required for tocotrienol biosynthesis in plants.

SUMMARY OF THE INVENTION

Compositions and methods for the alteration of the tocol content and composition of plants are provided. The compositions comprise novel nucleotide molecules comprising nucleotide sequences for HGGT. The compositions can be used to transform plants to manipulate the synthetic pathway for tocol compounds.

Transformed plants, plant cells, plant tissues, seed and grain are provided. Transformed plants of the invention find use in methods for improving grain or seed characteristics including, but not limited to, antioxidant level or activity.

Expression cassettes comprising sequences of the invention are provided. Isolated polypeptides encoded by the nucleotide sequences of the invention are also provided.

DETAILED DESCRIPTION

The problem to be solved is identifying the nucleic acids that encode polypeptides required for tocotrienol biosynthesis in plants. These polynucleotides may be used in plant cells and photosynthetic microbes to alter the tocols, such as tocotrienols, produced in the cells. More specifically, the polynucleotides of the instant invention may be used to significantly increase the content of vitamin E-related antioxidants such as tocotrienol in edible tissues of vegetable, fruit, and agronomic crop plants, including grains such as corn and soybean seed. The availability of nucleic acid sequences encoding all or a portion of the enzyme homogentisate geranylgeranyl transferase (HGGT) would facilitate studies to better understand tocotrienol biosynthesis in plants and provide genetic tools to alter tocotrienol metabolism. The present invention has solved this problem by providing nucleotide and deduced amino acid sequences corresponding to novel HGGT polynucleotides and corresponding polypeptides from barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*).

The HGGT-catalyzed reaction is analogous to the first step in tocopherol biosynthesis, which involves the condensation of homogentisate and phytyl-PP to form 2-methyl-6-phytylbenzoquinol (Soll, J. et al., supra). The latter reaction is catalyzed by the enzyme homogentisate phytyltransferase (HPT). cDNAs encoding HPT from a number of plant species have previously been disclosed in World Patent Application WO 00/68393. Given the similarity in their substrates and activity, one can hypothesize that HGGT is a divergent, but related form of HPT. It is likely that HGGT is the only specialized enzyme in the tocotrienol biosynthetic pathway. Methylation and cyclization reactions that convert the HGGT product 2-methyl-6-geranylgeranylbenzoquinol into tocotrienols are likely catalyzed by enzymes that are shared between the tocopherol and tocotrienol biosynthetic pathways (Schultz, G. et al. (1985) *Physiol. Plant* 64:123–129). As such, expression of HGGT will be sufficient to confer tocotrienol biosynthesis to a plant, plant tissue, a cell from plant tissue or a photosynthetic microbe that does not normally produce tocotrienols.

Recently, genes or cDNAs for HPT have been identified and characterized from the cyanobacterium Synechocystis sp. PCC 6803 and *Arabidopsis thaliana* based on their sequence similarity to chlorophyll synthases (Schledz, M. et al. (2001) *FEBS*499:15–20; Collakova, E. and DellaPenna, D. (2001) *Plant Physiol.* 127:1113–1124). cDNAs for homologs of the Arabidopsis HPT have also been identified from wheat, rice, corn and soybean (Collakova, E. and DellaPenna, D. (2001) *Plant Physiol.* 127:1113–1124).

The invention is drawn to compositions and methods for altering tocols. The compositions and methods find use in improving the antioxidant quality of grain for use as food for humans and feed for livestock. Furthermore, the tocols can be extracted, purified or further altered via processing. As

used herein, "grain" means the mature seed produced by commercial growers for purposes other than reproducing the species and/or immature seed as an integral part of whole plant corn harvested for silage. As used herein, grain includes plant parts commonly categorized as a fruit, nut or vegetable.

As used herein, "wild-type" refers to untransformed organisms and descendants of untransformed organisms.

The term "tocol" refers generally to any of the tocopherol and tocotrienol molecular species (e.g., α-, β-, γ-, and δ-) that are known to occur in biological systems. The term "tocol content" refers to the total amount of tocopherol and tocotrienol in a whole plant, tissue, or cell or in a microbial host. The term "tocol composition" refers both to the ratio of the various tocols produced in any given biological system and to altered characteristics, such as antioxidant activity, of any one tocol compound. When the alteration of tocols is taught or claimed herein, such alteration can be to tocol content and/or tocol composition. When an increase of tocols is taught or claimed herein, such increase refers to an increase of tocol content and/or an increase of tocol activity.

The term "tocotrienol" refers generally to any of the tocotrienol molecular species (e.g., α-, β-, γ-, and δ-) that are known to occur in biological systems. The term "tocotrienol content" refers to the total amount of tocotrienol in a whole plant, tissue, or cell or in a microbial host. The term "tocotrienol composition" refers both to the ratio of the various tocotrienols produced in any given biological system and to altered characteristics, such as antioxidant activity, of any one tocotrienol compound. When the alteration of a tocotrienol is taught or claimed herein, such alteration can be to tocotrienol content and/or tocotrienol composition. When an increase of tocotrienols is taught or claimed herein, such increase refers to an increase of tocotrienol content and/or an increase of tocotrienol activity.

The term "homogentisate phytyltransferase" or "HPT" refers to the enzyme that catalyzes the condensation of homogentisate (or homogentisic acid) and phytyl pyrophosphate (or phytyl diphosphate). This reaction is believed to be the committed step in tocopherol biosynthesis. Other names that have been used to refer to this enzyme include "homogentisate phytyl pyrophosphate prenyltransferase" and "homogentisate phytyl diphosphate prenyltransferase". The shortened version phytyl/prenyl transferase is also used.

The term "homogentisate geranylgeranyl transferase" or "HGGT" refers to the enzyme that catalyzes the condensation of homogentisate (or homogentisic acid) and geranylgeranyl pyrophosphate (or geranylgeranyl diphosphate). This reaction is an important step in tocotrienol biosynthesis and can result in the alteration of the tocol content and/or composition.

The invention provides isolated nucleotide molecules comprising nucleotide sequences encoding HGGT. Also provided are isolated polypeptides encoded by such nucleotide sequences. The nucleotide sequences find use in methods for altering tocols and tocotrienols in a biological system such as a plant. The methods include improving the antioxidant activity of grain, altering tocotrienols in a plant or part thereof, and improving tocols in a host. The methods comprise transforming a plant or host with at least one nucleotide construct comprising at least a portion of at least one nucleotide sequence of the invention. If desired, the nucleotide construct may additionally comprise an operably linked promoter that drives expression in the plant of interest. Such a nucleotide construct can be used to increase the expression of HGGT.

Among the many applications of improved tocols, tocotrienols and antioxidant activity are improved storage of grain, improved stability of oil extracted from grain, health benefits to humans and animals consuming the grain, and the production of novel tocols or tocotrienols for cosmetic, industrial and/or nutraceutical use. It is also known that the presence of tocols in plant vegetative green tissue such as leaf tissue is necessary to protect the plant from the photo-oxidative damage induced directly and indirectly by the production of free oxygen radicals in the chloroplast during oxygenic photosynthesis. It is therefore likely that ectopic expression of tocotrienols in green plant tissue, such as leaf tissue, in addition to the normal tocopherol content of the leaf will lead to an increase ability to withstand such photo-oxidative damage, and thus lead to an increase in the photosynthetic capacity of the plant. This would translate to an increase in harvestable yield for the plant over the entire growing season.

The nucleotide constructs of the invention comprise at least a portion of a nucleotide sequence of the invention. The nucleotide construct of the invention may additionally comprise at least one promoter that drives expression in a host or plant. Preferred promoters include, for corn, an embryo specific promoter such as promoters for the 16 kDa and 18 kDa oleosin genes, an endosperm specific promoter, such as the promoter for the 10 kDa zein gene, and a vegetative promoter such as promoters for ubiquitin genes.

A nucleotide construct of the invention comprises at least a portion of one nucleotide sequence of the invention. Preferably, such a nucleotide construct additionally comprises an operably linked promoter that drives expression in a plant. If desired, two or more of such nucleotide sequences may be linked or joined together to form one polynucleotide molecule, and such a polynucleotide may be used to transform a plant. For example, a nucleotide construct comprising a nucleotide sequence encoding a HGGT can be linked with another nucleotide sequence encoding the same or another HGGT. Nucleotide sequences encoding both HGGT and HPT may also be linked in a nucleotide construct. Similarly, the two nucleotide sequences can be provided on different nucleotide constructs, and each of the separate nucleotide sequences can be operably linked to a promoter that drives expression in a plant. For example, a construct may be used that increases total HGGT activity and decreases total HPT activity, thereby resulting in shunting the pathway towards the production of tocotrienols and decreased production of tocopherols. The alternative strategy may also be used. If separate nucleotide constructs are employed for the HGGT nucleotide sequence and an HPT nucleotide sequence, two individual plants may be transformed with the nucleotide constructs, and the plants may then be crossed to produce progeny having the desired genotype of both the HGGT and HPT nucleotide sequences.

Similarly, a construct to down-regulate the geranylgeranyl reductase responsible for producing phytol pyrophosphophosphate, one of the precursors for tocopherol biosynthesis, may be linked in cis with a construct to express HGGT. The result of this manipulation would be an increased pool size of geranylgeranyl-pyrophosphate and a corresponding increase of flux into the tocotrienol biosynthetic pathway.

The methods of the present invention can be employed to alter tocols or tocotrienols in any plant or part thereof, and antioxidant activity may thereby be altered. Plants that may be used in the invention include field crops (e.g., alfalfa, barley, bean, maize, cotton, flax, pea, rape, rice, rye, safflower, sorghum, oats, millet, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli,

cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); and fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon) and *Arabidopsis*. Some methods of the invention involve altering the antioxidant levels in grain and other parts of a plant that may be subjected to post-harvest processing or can be used as food source for humans, livestock and other animals, such as pets. With post-harvest processing, the tocols or tocotrienols so produced can be a valuable source of recovery for millers and other processors.

Methods for assessing tocopherol content and tocopherol composition (including tocopherol activity) are known in the art. Tocopherol content and composition may be measured by HPLC in combination with fluorescence detection. Such methods are described in Example 2 and in numerous literature references (e.g., Kamal-Eldi A., Gorgen S., Pettersson J., Lampi A. M. (2000) J Chromatogr A 881: 217–227; Bonvehi J. S., Coll F. V., Rius I. A. (2000) J. AOAC Intl. 83:627–634; Goffman F. D. and Böhme T. (2001) J Agric. Food Chem. 49:4990–4994). Such methods typically involve the resolution of tocopherol molecular species contained in complex mixtures by use of a normal or reverse phase HPLC matrix. Eluted tocopherol molecular species are then detected by fluorescence of the chromanol head group with an excitation wavelength typically in the range of 290 to 295 nm and an emission wavelength typically in the range of 325 to 335 nm. Using this methodology, the composition of a tocopherol mixture can be determined by comparing the retention times of separated molecular species with those of known standards. The content of each tocopherol molecular species can be measured by the relative intensity of its fluorescence emission at the selected wavelength. The absolute amount of each tocopherol species can be determined by measuring the intensity of fluorescence emission relative to that of an internal standard, which is added in a known amount to the tocopherol mixture prior to HPLC analysis. A suitable internal standard can include a tocopherol analog that is not normally found in nature (e.g., 5,7-dimethyltocol) or a naturally occurring tocopherol molecular species that is not present in a given tocopherol mixture. The total tocopherol content of a complex mixture of compounds can be derived by summing the absolute amount of each of the component tocopherol molecular species as determined by HPLC analysis.

Methods for assessing tocotrienol content and tocotrienol composition (including tocotrienol activity) are known in the art. Tocotrienol content and composition may be measured by HPLC using methods described above for the analysis of tocopherol content and composition. Using HPLC techniques described in Example 2 and elsewhere (e.g., Podda M., Weber C., Traber M. G., Packer L. (1996) J. Lipid Res. 37:893–901), tocotrienol molecular species can be readily resolved from tocopherol molecular species in a complex mixture. The occurrence and structural identification of tocotrienols in a complex mixture can be determined by gas chromatography-mass spectrometry as described by Frega N., Mozzon M., and Bocci F. (1998) J. Amer. Oil Chem. Soc. 75:1723–1728.

In addition, lipophilic antioxidant activity may be measured by assays including the inhibition of the coupled auto-oxidation of linoleic acid and β-carotene and oxygen radical absorbance capacity (ORAC) as described elsewhere (Serbinova E. A. and Packer L. (1994) Meth. Enzymol. 234:354–366; Emmons C. L., Peterson D. M., Paul G. L. (1999) J. Agric. Food Chem. 47:4894–4898); Huang D et al (2002) J. Agric. Food Chem.) in the press. Such methods typically involve measuring the ability of antioxidant compounds (i.e., tocols) in test materials to inhibit the decline of fluorescence of a model substrate (fluorescein, phycoerythrin) induced by a peroxyl radical generator (2',2'-azobis [20amidinopropane]dihydrochloride).

The nucleotide constructs of the invention can also be used to decrease or suppress the expression of endogenous HGGT in a plant. Decreasing the expression of HGGT involved in the production of tocotrienol can serve to shift the tocol pathway toward the production of tocopherol. A plant can be transformed with the HGGT nucleotide sequences in the sense orientation for co-suppression or sense suppression of gene expression. Alternatively, the plant can be transformed with the HGGT nucleotide sequences in the antisense orientation for antisense suppression. Expression of HGGT polypeptides can also be suppressed by modifying genomic sequences in a plant by chimeraplasty. Generally, such modifications will alter the amino acid sequence of the polypeptides encoded by the genomic sequence as to reduce or eliminate the activity of a HGGT in a plant, particularly in a seed.

Compositions of the invention include nucleotide sequences encoding HGGT polypeptides that are involved in regulating tocols or tocotrienols. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NOS: 2, 4, 6, 8 and 10. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID NOS: 1, 3, 5, 7, and 9, and fragments and variants thereof.

The invention encompasses isolated or substantially purified nucleic acid or polypeptide compositions. An "isolated" or "purified" nucleic acid molecule or polypeptide, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, 0.3 kb or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating polypeptide. When the polypeptide of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, 5%, 3% or 1% (by dry weight) of chemical precursors or non-polypeptide-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and polypeptides encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence. Fragments of a nucleotide sequence may encode polypeptide fragments that retain the

biological activity of the native protein and hence HGGT activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode polypeptides retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 30 nucleotides, about 50 nucleotides, about 70 nucleotides, about 100 nucleotides, about 150 nucleotides and up to the full-length nucleotide sequence encoding the polypeptides of the invention.

A fragment of a HGGT nucleotide sequence that encodes a biologically active portion of an HGGT polypeptide of the invention will encode at least 15, 25, 30, 50, 75, 100, or 125 contiguous amino acids, or up to the total number of amino acids present in a full-length HGGT polypeptide of the invention (for example, 407, 408, 404, 380 and 361 amino acids for SEQ ID NO: 2, 4, 6, 8 and 10 respectively). Fragments of a HGGT nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of an HGGT polypeptide.

Thus, a fragment of an HGGT nucleotide sequence may encode a biologically active portion of an HGGT polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an HGGT polypeptide can be prepared by isolating a portion of one of the HGGT nucleotide sequences of the invention, expressing the encoded portion of the HGGT polypeptide (e.g., by recombinant expression in vitro) and assessing the activity of the encoded portion of the HGGT polypeptide. Conserved motifs for HGGT sequences are identified in SEQ. ID Nos: 56–66.

Nucleic acid molecules that are fragments of an HGGT nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, or 700 nucleotides, or up to the number of nucleotides present in a full-length HGGT nucleotide sequence disclosed herein (for example, 1457, 1365, 1242, 1730, and 1769 nucleotides for SEQ ID NO: 1, 3, 5, 7 and 9, respectively). The coding sequences for the conserved motifs identified in SEQ. ID Nos: 56–66 can also be easily identified from the HGGT sequences provided herein. The same is also true of degenerate sequences coding for the conserved motifs identified in SEQ. ID Nos: 56–66.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the HGGT polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode an HGGT polypeptide of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 80% generally at least about 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters. Optionally, variants will also encode for at least any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the conserved motifs identified in SEQ. ID Nos: 56–66.

By "variant" polypeptide is intended a polypeptide derived from the native polypeptide by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native polypeptide; deletion or addition of one or more amino acids at one or more sites in the native polypeptide; or substitution of one or more amino acids at one or more sites in the native polypeptide. Variant polypeptides encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native polypeptide, that is, HGGT activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native HGGT polypeptide of the invention will have at least about 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to the amino acid sequence for the native polypeptide as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a polypeptide of the invention may differ from that polypeptide by as few as 1–15 amino acid residues, as few as 1–10, such as 6–10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue. Optionally, variants will also comprise at least any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 of the conserved motifs identified in SEQ. ID Nos: 56–66.

The polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the HGGT polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488–492; Kunkel et al. (1987) *Methods in Enzymol.* 154:367–382; U.S. Pat. No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide of interest may be found in the model of Dayhoff et al. (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the polypeptides of the invention encompass both naturally occurring polypeptides as well as variations and modified forms thereof. Such variants will continue to possess the desired HGGT activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the polypeptide sequences encompassed herein are not expected to produce radical changes in the characteristics of the polypeptide. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by assays for HGGT activity.

Variant nucleotide sequences and polypeptides also encompass sequences and polypeptides derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different HGGT coding sequences can be manipulated to create a new HGGT polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the HGGT polynucleotides of the invention and/or other HGGT genes to obtain a new gene coding for a polypeptide with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747–10751; Stemmer (1994) *Nature* 370:389–391; Crameri et al. (1997) *Nature Biotech.* 15:436–438; Moore et al. (1997) *J. Mol. Biol.* 272:336–347; Zhang et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:4504–4509; Crameri et al. (1998) *Nature* 391:288–291; and U.S. Pat. Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire HGGT nucleotide sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended polynucleotides derived from a common ancestral gene and which are found in different species as a result of speciation. Polynucleotides found in different species are considered orthologs when their nucleotide sequences and/or their encoded polypeptide sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.). See also Innis et al., eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

For clarification, "PCR" or "polymerase chain reaction" is a technique for the synthesis of large quantities of specific DNA segments, consists of a series of repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, Conn.). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the HGGT sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

For example, an entire HGGT sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding HGGT sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among HGGT sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Polynucleotide sequences useful as probes include the polynucleotide sequences encoding the conserved motifs set forth in SEQ. ID Nos: 56–66. Such probes may be used to amplify corresponding HGGT sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1× to 2×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1%

SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267–284: T_m=81.5° C.+16.6 (log M)+0.41 (% GC)–0.61 (% form)–500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1° C. for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ≧90% identity are sought, the T_m can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C. lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10° C. lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C. lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45° C. (aqueous solution) or 32° C. (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, N.Y.); and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

Isolated sequences that encode for a HGGT polypeptide and which hybridize under stringent conditions to the HGGT sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

Nucleotides (usually found in their T-monophosphate form) are often referred to herein by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "W" for A or T, "H" for A or C or T, "D" for A or G or T, "M" for A or C, "S" for C or G, "V" for A or C or G, "B" for C or G or T "I" for inosine, and "N" for A, C, G, or T.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides:(a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11–17; the local homology algorithm of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443–453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444–2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 872264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873–5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., U.S.A.). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. (1988) *Gene* 73:237–244 (1988); Higgins et al. (1989) *CABIOS* 5:151–153; Corpet et al. (1988) *Nucleic Acids Res.* 16:10881–90; Huang et a. (1992) *CABIOS* 8:155–65; and Pearson et al. (1994) *Meth. Mol. Biol.* 24:307–331. The ALIGN program is based on the algorithm of Myers and Miller (1988) supra. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul et al (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. BLAST nucleotide searches can be performed with the BLASTN program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a polypeptide of the invention. BLAST polypeptide searches can be performed with the BLASTX program, score=50, wordlength=3, to obtain amino acid sequences homologous to a polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that

detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for polypeptides) can be used. See http://www.ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443–453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for polypeptide sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

For purposes of the present invention, comparison of nucleotide or polypeptide sequences for determination of percent sequence identity to the HGGT sequences disclosed herein is preferably made using CLUSTAL with the following changes from the default parameters. For amino acid sequence comparisons a Gap Penalty of 10 and Gap Length Penalty of 10 was used for multiple alignments and a KTUPLE of 2, Gap Penalty of 3, Window of 5 and Diagonals Saved of 5 was used for pairwise alignments. For nucleotide sequence comparisons, a Gap Penalty of 10 and Gap Length Penalty of 10 was used for multiple alignments and a KTUPLE of 2, Gap Penalty of 5, Window of 4 and Diagonals Saved of 4 was used for pairwise alignments. Any equivalent program can also be used to determine percent sequence identity. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to polypeptides it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of polypeptides encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other

under stringent conditions. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C. to about 20° C. lower than the T_m, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443–453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "nucleotide constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the nucleotide constructs of the present invention encompass all nucleotide constructs that can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

Furthermore, it is recognized that the methods of the invention may employ a nucleotide construct that is capable of directing, in a transformed plant, the expression of at least one polypeptide, or at least one RNA, such as, for example, an antisense RNA that is complementary to at least a portion of an mRNA. Typically such a nucleotide construct is comprised of a coding sequence for a polypeptide or an RNA operably linked to 5' and 3' transcriptional regulatory regions. Alternatively, it is also recognized that the methods of the invention may employ a nucleotide construct that is not capable of directing, in a transformed plant, the expression of a polypeptide or an RNA.

In addition, it is recognized that methods of the present invention do not depend on the incorporation of the entire nucleotide construct into the genome, only that the plant or cell thereof is altered as a result of the introduction of the nucleotide construct into a cell. In one embodiment of the invention, the genome may be altered following the introduction of the nucleotide construct into a cell. For example, the nucleotide construct, or any part thereof, may incorporate into the genome of the plant. Alterations to the genome of the present invention include, but are not limited to, additions, deletions, and substitutions of nucleotides in the genome. While the methods of the present invention do not depend on additions, deletions, or substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprise at least one nucleotide.

The nucleotide constructs of the invention also encompass nucleotide constructs that may be employed in methods for altering or mutating a genomic nucleotide sequence in an organism, including, but not limited to, chimeric vectors, chimeric mutational vectors, chimeric repair vectors, mixed-duplex oligonucleotides, self-complementary chimeric oligonucleotides, and recombinogenic oligonucleobases. Such nucleotide constructs and methods of use, such as, for example, chimeraplasty, are known in the art. Chimeraplasty involves the use of such nucleotide constructs to introduce site-specific changes into the sequence of genomic DNA within an organism. See, U.S. Pat. Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821, and Beetham et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:8774–8778; herein incorporated by reference.

The HGGT sequences of the invention are provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a HGGT nucleotide sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two polypeptide coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the HGGT nucleotide sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'–3' direction of transcription, a transcriptional and translational initiation region, a HGGT polynucleotide sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of

HGGT in the plant, plant cell or other host. Thus, the phenotype of the plant, plant cell or other host is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau et al. (1991) *Mol. Gen. Genet* 262:141–144; Proudfoot (1991) *Cell* 64:671–674; Sanfacon et al. (1991) *Genes Dev.* 5:141–149; Mogen et al. (1990) *Plant Cell* 2:1261–1272; Munroe et al. (1990) *Gene* 91:151–158; Ballas et al. (1989) *Nucleic Acids Res.* 17:7891–7903; and Joshi et al. (1987) *Nucleic Acid Res.* 15:9627–9639.

Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1–11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Pat. Nos. 5,380,831, and 5,436,391, and Murray et al. (1989) *Nucleic Acids Res.* 17:477–498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:6126–6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie et al. (1995) *Gene* 165(2):233–238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9–20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) *Nature* 353:90–94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling et al. (1987) *Nature* 325:622–625); tobacco mosaic virus leader (TMV) (Gallie et al. (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237–256); and maize chlorotic mottle virus leader (MCMV) (Lommel et al. (1991) *Virology* 81:382–385). See also, Della-Cioppa et al. (1987) *Plant Physiol.* 84:965–968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, in vitro mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, chemically regulated, tissue-preferred, or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Pat. No. 6,072,050; the core CaMV 35S promoter (Odell et al. (1985) *Nature* 313:810–812); rice actin (McElroy et al. (1990) *Plant Cell* 2:163–171); ubiquitin (Christensen et al. (1989) *Plant Mol. Biol.* 12:619–632 and Christensen et al. (1992) *Plant Mol. Biol.* 18:675–689); pEMU (Last et al. (1991) *Theor. Appl. Genet.* 81:581–588); MAS (Velten et al. (1984) *EMBO J.* 3:2723–2730); ALS promoter (U.S. Pat. No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Pat. Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

Chemically regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical inducible promoter, where application of the chemical induces gene expression, or a chemical repressible promoter, where application of the chemical represses gene expression. Chemical inducible promoters are known in the art and include, but are not limited to, the maize In2—2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemically regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:10421–10425 and McNellis et al. (1998) *Plant J.* 14(2):247–257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz et al. (1991) *Mol. Gen. Genet.* 227:229–237, and U.S. Pat. Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced HGGT expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto et al. (1997) *Plant J.* 12(2)255–265; Kawamata et al. (1997) *Plant Cell Physiol.* 38(7):792–803; Hansen et al. (1997) *Mol. Gen Genet.* 254(3):337–343; Russell et al. (1997) *Transgenic Res.* 6(2):157–168; Rinehart et al. (1996) *Plant Physiol.* 112(3):1331–1341; Van Camp et al. (1996) *Plant Physiol.* 112(2):525–535; Canevascini et al. (1996) *Plant Physiol.* 112(2):513–524; Yamamoto et al. (1994) *Plant Cell Physiol.* 35(5):773–778; Lam (1994) *Results Probl. Cell Differ.* 20:181–196; Orozco et al. (1993) *Plant Mol Biol.* 23(6): 1129–1138; Matsuoka et al. (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586–9590; and Guevara-Garcia et al. (1993) *Plant J.* 4(3):495–505. Such promoters can be modified, if necessary, for weak expression.

Leaf-specific promoters are known in the art. See, for example, Yamamoto et al. (1997) *Plant J.* 12(2):255–265; Kwon et al. (1994) *Plant Physiol.* 105:357–67; Yamamoto et al. (1994) *Plant Cell Physiol.* 35(5):773–778; Gotor et al. (1993) *Plant J.* 3:509–18; Orozco et al. (1993) *Plant Mol. Biol.* 23(6):1129–1138; and Matsuoka et al. (1993) Proc. Natl. Acad. Sci. USA 90(20):9586–9590.

Root-specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example,

Hire et al. (1992) *Plant Mol. Biol.* 20(2): 207–218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051–1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger et al. (1990) *Plant Mol. Biol.* 14(3):433–443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao et al. (1991) *Plant Cell* 3(1):11–22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz et al. (1990) *Plant Cell* 2(7):633–641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69–76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri et al. (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2): 343–350). The TR1' gene, fused to nptII (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster et al. (1995) *Plant Mol. Biol.* 29(4): 759–772); and rolB promoter (Capana et al. (1994) *Plant Mol. Biol.* 25(4):681–691. See also U.S. Pat. Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson et al. (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); and milps (myo-inositol-1-phosphate synthase); (see WO 00/11177, herein incorporated by reference). The 27 kDa gamma-zein promoter is a preferred endosperm-specific promoter. The maize globulin-1 and oleosin promoters are preferred embryo-specific promoters. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, promoters of the 15 kDa beta-zein, 22 kDa alpha-zein, 27 kDa gamma-zein, waxy, shrunken 1, shrunken 2, globulin 1 and oleosin genes. See also WO 00/12733, where seed-preferred promoters from end1 and end2 genes are disclosed; herein incorporated by reference.

In a preferred embodiment, the nucleic acids of interest are targeted to the chloroplast for expression. In this manner, where the nucleic acid of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a nucleic acid encoding a transit peptide to direct the gene product of interest to the chloroplasts or other plastids. Such transit peptides are known in the art. See, for example, Von Heijne et al. (1991) *Plant Mol. Biol. Rep.* 9:104–126; Clark et al. (1989) *J. Biol. Chem.* 264:17544–17550; Della-Cioppa et al. (1987) *Plant Physiol.* 84:965–968; Romer et al. (1993) *Biochem. Biophys. Res. Commun.* 196:1414–1421; and Shah et al. (1986) *Science* 233:478–481.

The HGGT polypeptides of the invention can be targeted to specific compartments within the plant cell. Methods for targeting polypeptides to a specific compartment are known in the art. Generally, such methods involve modifying the nucleotide sequence encoding the polypeptide in such a manner as to add or remove specific amino acids from the polypeptide encoded thereby. Such amino acids comprise targeting signals for targeting the polypeptide to a specific compartment such as, for example, a the plastid, the nucleus, the endoplasmic reticulum, the vacuole, the mitochondrion, the peroxisome, the Golgi apparatus, and for secretion from the cell. Targeting sequences for targeting a polypeptide to a specific cellular compartment, or for secretion, are known to those of ordinary skill in the art. Chloroplast-targeting or plastid-targeting sequences are known in the art and include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (de Castro Silva Filho et al. (1996) *Plant Mol. Biol.* 30:769–780; Schnell et al. (1991) *J. Biol. Chem.* 266(5):3335–3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer et al. (1990) *J. Bioenerg. Biomemb.* 22(6):789–810); tryptophan synthase (Zhao et al. (1995) *J. Biol. Chem.* 270(11):6081–6087); plastocyanin (Lawrence et al. (1997) *J. Biol. Chem.* 272(33):20357–20363); chorismate synthase (Schmidt et al. (1993) *J. Biol. Chem.* 268(36):27447–27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa et al. (1988) *J. Biol. Chem.* 263:14996–14999). See also Von Heijne et al. (1991) *Plant Mol. Biol. Rep.* 9:104–126; Clark et al. (1989) *J. Biol. Chem.* 264:17544–17550; Della-Cioppa et al. (1987) *Plant Physiol.* 84:965–968; Romer et al. (1993) *Biochem. Biophys. Res. Commun.* 196:1414–1421; and Shah et al. (1986) *Science* 233:478–481.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase, as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:50–511; Christopherson et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6314–6318; Yao et al. (1992) *Cell* 71:63–72; Reznikoff (1992) *Mol. Microbiol.* 6:2419–2422; Barkley et al. (1980) in *The Operon*, pp. 177–220; Hu et al. (1987) *Cell* 48:555–566; Brown et al. (1987) *Cell* 49:603–612; Figge et al. (1988) *Cell* 52:713–722; Deuschle et al. (1989) *Proc. Natl. Acad. Aci. USA* 86:5400–5404; Fuerst et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:2549–2553; Deuschle et al. (1990) *Science* 248:480–483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1917–1921; Labow et al. (1990) *Mol. Cell. Biol.* 10:3343–3356; Zambretti et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:3952–3956; Baim et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:5072–5076; Wyborski et al. (1991) *Nucleic Acids Res.* 19:4647–4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143–162; Degenkolb et al. (1991) *Antimicrob. Agents Chemother.* 35:1591–1595; Kleinschnidt et al. (1988) *Biochemistry* 27:1094–1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen et al. (1992)

Proc. Natl. Acad. Sci. USA 89:5547–5551; Oliva et al. (1992) *Antimicrob. Agents Chemother.* 36:913–919; Hlavka et al. (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill et al. (1988) *Nature* 334:721–724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention

The invention involves transforming host cells with the nucleotide constructs of the invention. Generally, the nucleotide construct will comprise a HGGT nucleotide sequence of the invention, either a full length sequence or functional fragment thereof, operably linked to a promoter that drives expression in the host cell of interest. Host cells include, but are not limited to: plant cells; animal cells; fungal cells, particularly yeast cells; and bacterial cells.

The methods of the invention involve introducing a nucleotide construct into a plant. By "introducing" is intended presenting to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a nucleotide construct to a plant, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

By "stable transformation" is intended that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleotide construct introduced into a plant does not integrate into the genome of the plant.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway et al. (1986) *Biotechniques* 4:320–334), electroporation (Riggs et al. (1986) *Proc. Natl. Acad. Sci. USA* 83:5602–5606, *Agrobacterium*-mediated transformation (Townsend et al., U.S. Pat. No. 5,563,055; Zhao et al., U.S. Pat. No. 5,981,840), direct gene transfer (Paszkowski et al. (1984) *EMBO J.* 3:2717–2722), and ballistic particle acceleration (see, for example, Sanford et al., U.S. Pat. No. 4,945,050; Tomes et al., U.S. Pat. No. 5,879,918; Tomes et al., U.S. Pat. No. 5,886,244; Bidney et al., U.S. Pat. No. 5,932,782; Tomes et al. (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe et al. (1988) *Biotechnology* 6:923–926); and Lec1 transformation (WO 00/28058). Also see Weissinger et al. (1988) *Ann. Rev. Genet* 22:421–477; Sanford et al. (1987) *Particulate Science and Technology* 5:27–37 (onion); Christou et al. (1988) *Plant Physiol.* 87:671–674 (soybean); McCabe et al. (1988) *Bio/Technology* 6:923–926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P: 175–182 (soybean); Singh et al. (1998) *Theor. Appl. Genet* 96:319–324 (soybean); Datta et al. (1990) *Biotechnology* 8:736–740 (rice); Klein et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:4305–4309 (maize); Klein et al. (1988) *Biotechnology* 6:559–563 (maize); Tomes, U.S. Pat. No. 5,240,855; Buising et al., U.S. Pat. Nos. 5,322,783 and 5,324,646; Tomes et al. (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein et al. (1988) *Plant Physiol.* 91:440–444 (maize); Fromm etal. (1990) *Biotechnology* 8:833–839 (maize); Hooykaas-Van Slogteren et al. (1984) *Nature* (London) 311:763–764; Bowen et al., U.S. Pat. No. 5,736,369 (cereals); Bytebier et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:5345–5349 (Liliaceae); De Wet et al. (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman et al. (Longman, N.Y.), pp. 197–209 (pollen); Kaeppler et al. (1990) *Plant Cell Reports* 9:415–418 and Kaeppler et al. (1992) *Theor. Appl. Genet* 84:560–566 (whisker-mediated transformation); D'Halluin et al. (1992) *Plant Cell* 4:1495–1505 (electroporation); Li et al. (1993) *Plant Cell Reports* 12:250–255 and Christou and Ford (1995) *Annals of Botany* 75:407–413 (rice); Osjoda et al. (1996) *Nature Biotechnology* 14:745–750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The nucleotide constructs of the invention may also be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. It is recognized that a HGGT of the invention may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis in vivo or in vitro to produce the desired recombinant polypeptide. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing nucleotide constructs into plants and expressing a polypeptide encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Pat. Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

Methods for transformation of chloroplasts are known in the art. See, for example, Svab et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:8526–8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913–917; Svab and Maliga (1993) *EMBO J.* 12:601–606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:7301–7305.

The nucleic acids of interest to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the nucleic acids of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Pat. No. 5,380,831, herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al. (1986) *Plant Cell Reports* 5:81–84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

Transformed plants include those plants directly transformed as provided herein, as well as plants that have the directly transformed plants in their pedigree and retain the change in genotype, such as the inclusion of the expression cassette, created by the original transformation.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (Lathyrus spp.), and members of the genus Cucumis such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesil*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellowcedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, barley, rice, sorghum, rye, millet, tobacco, etc.), more preferably cereal plants, yet more preferably corn, wheat, barley, rice, sorghum, rye and millet plants.

It is recognized that with these nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for a HGGT sequence can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a nucleotide construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Pat. Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The following examples are presented by way of illustration, not by way of limitation.

EXPERIMENTAL

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration, not by way of limitation. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

EXAMPLE 1

Composition of cDNA Libraries;

Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) tissues were prepared. The cDNA libraries representing mRNAs from barley (*Hordeum vulgare*) and rice (*Oryza sativa*) developing seeds are described in Examples 2 and 4, respectively. The characteristics of the corn (*Zea mays*) and wheat (*Triticum aestivum*) libraries are described in Table 1 below.

TABLE 1

cDNA Libraries from Corn and Wheat

Library	Tissue	Clone
cco1n	Corn (*Zea mays*) cob of 67 day old plants grown in green house*	cco1n.pk087.l17: fis
wdk2c	Wheat (*Triticum aestivum*) developing kernel, 7 days after anthesis	wdk2c.pk012.f2: fis
p0058	Sweet Corn (*Zea mays*) hybrid (Honey N Pearl) shoot culture. It was initiated on 2/28/96 from seed derived meristems. The culture was maintained on 273 N medium.	p0058.chpbj67r: fis

*These libraries were normalized essentially as described in U.S. Pat. No. 5,482,845 (the contents of which are hereby incorporated by reference).

cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be intro-

duced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, Calif.). The Uni-ZAP™ M XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBluescript. In addition, the cDNAs may be introduced directly into precut Bluescript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al. (1991) *Science* 252:1651–1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

Full-insert sequence (FIS) data is generated utilizing a modified transposition protocol. Clones identified for FIS are recovered from archived glycerol stocks as single colonies, and plasmid DNAs are isolated via alkaline lysis. Isolated DNA templates are reacted with vector primed M13 forward and reverse oligonucleotides in a PCR-based sequencing reaction and loaded onto automated sequencers. Confirmation of clone identification is performed by sequence alignment to the original EST sequence from which the FIS request is made.

Confirmed templates are transposed via the Primer Island transposition kit (PE Applied Biosystems, Foster City, Calif.) which is based upon the *Saccharomyces cerevisiae* Ty1 transposable element (Devine and Boeke (1994) *Nucleic Acids Res.* 22:3765–3772). The in vitro transposition system places unique binding sites randomly throughout a population of large DNA molecules. The transposed DNA is then used to transform DH10B electro-competent cells (Gibco BRL/Life Technologies, Rockville, Md.) via electroporation. The transposable element contains an additional selectable marker (named DHFR; Fling and Richards (1983) *Nucleic Acids Res.* 11:5147–5158), allowing for dual selection on agar plates of only those subclones containing the integrated transposon. Multiple subclones are randomly selected from each transposition reaction, plasmid DNAs are prepared via alkaline lysis, and templates are sequenced (ABI Prism dye-terminator ReadyReaction mix) outward from the transposition event site, utilizing unique primers specific to the binding sites within the transposon.

Sequence data is collected (ABI Prism Collections) and assembled using Phred/Phrap (P. Green, University of Washington, Seattle). Phrep/Phrap is a public domain software program which re-reads the ABI sequence data, re-calls the bases, assigns quality values, and writes the base calls and quality values into editable output files. The Phrap sequence assembly program uses these quality values to increase the accuracy of the assembled sequence contigs. Assemblies are viewed by the Consed sequence editor (D. Gordon, University of Washington, Seattle).

In some of the clones the cDNA fragment corresponds to a portion of the 3'-terminus of the gene and does not cover the entire open reading frame. In order to obtain the upstream information one of two different protocols are used. The first of these methods results in the production of a fragment of DNA containing a portion of the desired gene sequence while the second method results in the production of a fragment containing the entire open reading frame. Both of these methods use two rounds of PCR amplification to obtain fragments from one or more libraries. The libraries some times are chosen based on previous knowledge that the specific gene should be found in a certain tissue and some times are randomly-chosen. Reactions to obtain the same gene may be performed on several libraries in parallel or on a pool of libraries. Library pools are normally prepared using from 3 to 5 different libraries and normalized to a uniform dilution. In the first round of amplification both methods use a vector-specific (forward) primer corresponding to a portion of the vector located at the 5'-terminus of the clone coupled with a gene-specific (reverse) primer. The first method uses a sequence that is complementary to a portion of the already known gene sequence while the second method uses a gene-specific primer complementary to a portion of the 3'-untranslated region (also referred to as UTR). In the second round of amplification a nested set of primers is used for both methods. The resulting DNA fragment is ligated into a pBluescript vector using a commercial kit and following the manufacturer's protocol. This kit is selected from many available from several vendors including Invitrogen (Carlsbad, Calif.), Promega Biotech (Madison, Wis.), and Gibco-BRL (Gaithersburg, Md.). The plasmid DNA is isolated by alkaline lysis method and submitted for sequencing and assembly using Phred/Phrap, as above.

EXAMPLE 2

Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Barley Seed

In an attempt to identify cDNAs for homogentisate geranylgeranyl transferase (HGGT), degenerate PCR oligonucleotides were designed based on partially conserved domains in amino acid sequences deduced from homogentisate phytyl transferase (HPT) cDNAs disclosed in WO 00/68393 (the contents of which are hereby incorporated by reference). Degenerate sense and antisense oligonucleotides were designed that consist of the following sequences: 5'-TAYRTNGTNGGNHTNAAYCA-3' (SEQ ID NO:20) and 5'-GCRTARAANARNTTCCADATRAA-3' (SEQ ID NO:22). These oligonucleotides were designated HPT5' (SEQ ID NO:20) and HPT3' (SEQ ID NO:22), respectively, and correspond to the amino acid sequences: Y(I/V)VG(I/L/F/M)NQ (SEQ ID NO:21) and FIW(K/N)(I/L/M)FYA (SEQ ID NO:23).

It is known that tocotrienols are enriched in the seed endosperm of monocotyledonous plants, including barley (*Hordeum vulgare*) (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129–131; Qureshi, A. A. et al., (1986) *J. Biol. Chem.* 261:10544–10550). Such tissues therefore represent likely sources of expressed genes for the tocotrienol biosynthetic enzyme HGGT. As a first step towards the identification of an HGGT cDNA, total RNA was isolated from developing seeds of barley (cultivar Barsoy) using Trizol reagent (Life Technologies) according to the manufacturer's protocol. First strand cDNA was then prepared from 2 μg of the isolated RNA by using oligo-dT priming and Superscript II reverse transcriptase (Life Technologies) in a 25 μL reaction as described in the manufacturer's protocol. PCR amplification was then conducted using Advantage cDNA polymerase mix (Clontech) and 2 μL of the first strand

cDNA synthesis reaction as template in a total volume of 50 μL. Oligonucleotides HPT5' (SEQ ID NO: 20) and HPT3' (SEQ ID NO: 22) were included in the amplification reaction as sense and antisense primers. Forty cycles of amplification were conducted with annealing and extension temperatures of 50° C. and 72° C., respectively. The resulting PCR products displayed an approximate size of 700 nucleotides, as expected for the coding sequence of HPT-related enzymes. PCR products were subsequently subcloned into the vector pPCR-Script AMP (Stratagene) according to the manufacturer's protocol and transformed into *E. coli* DH10B cells (Gibco-BRL). Nucleotide sequence was obtained from the cDNA inserts of plasmids from twelve of the resulting colonies. The sequence of one cDNA insert (SEQ ID NO: 11) was found to encode 234 amino acids of an HPT-related polypeptide (SEQ ID NO: 12) that shared 58 to 61% identity with the analogous portion of HPT polypeptides from Arabidopsis (SEQ ID NO: 13), soybean (SEQ ID NO: 14), rice (SEQ ID NO: 15) and maize (SEQ ID NO: 16) that were disclosed in WO 00/68393. Over the same portion of their amino acid sequences, the *Arabidopsis*, soybean, rice and maize HPTs share >75% identity. Thus, the partial cDNA sequence identified from barley seeds encoded a divergent form of HPT that was subsequently determined to correspond to the polynucleotide sequence for HGGT.

In order to establish the function of the polypeptide encoded by the partial cDNA from barley seed, the complete 5' and 3' ends of the cDNA were amplified from a barley developing seed cDNA library using nested PCR. As used herein, the term "nested PCR" refers to a polymerase chain reaction (PCR) technique in which the product or products of a PCR reaction are reamplified by using an oligonucleotide primer combination in which one or both primers correspond to a portion of the target DNA that lies within the sequence amplified in the initial reaction. For the library construction, polyA$^+$-RNA was enriched from the developing seed total RNA described above using the QuickPrep mRNA purification kit (Pharmacia Biotech) according to the manufacturer's protocol. cDNA inserts were prepared from the polyA$^+$RNA using a Uni-ZAP XR cDNA synthesis kit (Stratagene) and cloned into the EcoRI/XhoI sites of the pBluescript SK(+) vector as previously described (Cahoon et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:12935–12940). Approximately 200 ng of the resulting plasmid library was used as template in nested PCR reactions described below. The reactions were conducted in 50 –μL volumes, and amplification was achieved using Advantage cDNA polymerase mix (Clontech) with an annealing temperature of 55° C. Reactions contained primer pairs that were designed based on sequence from the pBluescript SK(+) vector and from the partial cDNA amplified from barley seed (SEQ ID NO: 11). Thirty-five cycles were conducted in each of the PCR reactions described below. For amplification of the complete 5' end of the putative barley HGGT cDNA, reactions were performed with the following sense and antisense primers: 5'-AAATTAACCCTCACTAAAGGG-3' (modified T3 primer) (SEQ ID NO: 24) and 5'-ATACATGATGCAGCGAGGAGC-3' (SEQ ID NO:25). The unpurified products of this reaction were diluted six-fold, and 1 μL of this dilution was used as template in a second reaction that contained the following nested sense and antisense primers: 5'-CTCTAGAACTAGTGGATCCC-3' (modified SK primer) (SEQ ID NO: 26) and 5'-GTATTCCTATGCTAAAGCTC-3' (SEQ ID NO: 27). For amplification of the complete 3'end of the putative barley HGGT cDNA, reactions were conducted with the following sense and antisense primers: 5'-GAATTTTCAGTAGCAACTGG-3' (SEQ ID NO: 28) and 5'-GTAAAACGACGGCCAGT-3' (M13-20 primer) (SEQ ID NO: 29). The unpurified products of this reaction were diluted six-fold, and 1 μL of this dilution was used as template in a second reaction that contained the following nested sense and antisense primers: 5'-CTCCTCGCTGCATCATGTATC-3' (SEQ ID NO: 30) and 5'-GTAATACGACTCACTATAGGGC-3' (T7 primer) (SEQ ID NO: 31). The products of the nested reactions above were subcloned into the vector PGEM-T Easy (Promega), and transformed into *E. coli* DH10B cells. DNA sequences corresponding to the amplified 5' and 3' ends of the putative barley HGGT cDNA were then obtained from cDNA inserts of plasmids from several independent transformants. These sequences together with that from SEQ ID NO: 11 were assembled to generate the sequence of the full-length cDNA for the putative barley HGGT (SEQ ID NO: 1). The resulting full-length cDNA was designated "bdl2c.pk006.o2". The bdl2c.pk006.o2 cDNA (SEQ ID NO: 1) was found to encode a 407 amino acid polypeptide (SEQ ID NO: 2) that shares 45 to 47% identity with HPTs from Arabidopsis (SEQ ID NO: 13), soybean (SEQ ID NO: 14), rice (SEQ ID NO: 15) and maize (SEQ ID NO: 16) that were disclosed in WO 00/68393. By comparison, the *Arabidopsis*, soybean, rice, and maize HPTs share 61 to 69% identity with each other.

To examine the tissue-specific expression of the gene for the putative barley HGGT polypeptide, Northern blot analysis was conducted using polyA$^+$-enriched RNA isolated from leaf, roots and developing seeds of barley. For this experiment, total RNA was isolated from these tissues using the Trizol reagent (Life Technologies) according to the manufacturer's protocol, and polyA$^+$ RNA was then enriched from the total RNA extract using the PolyATract mRNA isolation system (Promega). Approximately 2 μg of polyA$^+$ from each tissue was electrophoresed in a 1% (w/v) agarose gel and then transferred from the gel to Bright Star-Plus nylon membrane (Ambion) using NorthernMax transfer buffer (Ambion). The RNA was fixed to the membrane by baking at 80° C. for 2 h. The membrane was rinsed with 2×SSC and then hybridized with ^{32}P-labeled probes for 18 h at 42° C. in NorthernMax hybridization buffer (Ambion). Probes were prepared from the full-length barley HPT-like cDNA (bdl2c.pk006.o2; SEQ ID NO: 1) and were labeled using random hexamer priming. Following incubation with probes, blots were washed for 15 min with 2×SSC/0.1% SDS at room temperature, then washed for a 15 min at room temperature with 0.2×SSC/0.1% SDS, and finally washed for 15 min at 42° C. with 0.2×SSC/0.1% SDS. Radioactivity on filters was detected by phosphorimaging. As a control for RNA loading, the Northern blot was re-hybridized with a labeled probe prepared from a barley α-tubulin cDNA, which corresponds to a constitutively expressed gene.

A Northern blot analysis to assess the expression of a nucleotide sequence encoding a barley HGGT was performed. Enriched polyA+ RNA isolated from leaf (L), root (R), and developing seed (S) of barley were analyzed. A barley a-tubulin gene, which is known to be a constitutively expressed gene in plants was analyzed using the same Northern blot as for HGGT gene expression and showed that the loading of polyA+ RNA from each tissue was approximately equal.

Expression of the gene for the putative barley HGGT was detected in developing seeds but was not detected in leaves and roots. The seed specific expression of the gene for the putative barley HGGT is consistent with the occurrence of

tocotrienols in barley seeds and the corresponding absence of these compounds from leaves and roots (Cahoon, unpublished observation).

To determine the function of the putative barley HGGT, the full-length bdl2c.pk006.o2 cDNA (SEQ ID NO: 1) encoding this enzyme was operably linked to the cauliflower mosaic virus 35S promoter and expressed in tobacco callus. The barley cDNA was initially amplified by PCR to generate flanking NcoI and EcoRI sites for cloning into the plant expression vector. The sequence of the sense oligonucleotide used in the amplification reaction was 5'-ttccatggCGAGGATGCAAGCCGTCACGG -3' (SEQ ID NO: 32), and the sequence of the antisense oligonucleotide was 5'-ttgaattcACACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 33). (Note: The bases in lower case contain the added restriction sites, which are underlined, and flanking sequence to facilitate restriction enzyme digestion.) Thirty cycles of PCR amplification were conducted in a 100 μL volume using Pfu polymerase (Stratagene) and approximately 300 ng of the barley developing seed cDNA library described above as template. The product of this reaction was purified by agarose electrophoresis, and then digested with the restriction enzymes NcoI and EcoRI. The amplified barley cDNA was then cloned into the corresponding sites of the plant expression vector pML63. A detailed description of vector pML63 has been previously disclosed in WO 00/11176 (the contents of which are hereby incorporated by reference). The resulting plasmid pBHGGT-35S contained the putative barley HGGT cDNA operably linked at its 5' end to the cauliflower mosaic virus 35S promoter and at its 3' end to termination sequence from the nopaline synthase (nos) gene. This expression cassette was then removed from pBHGGT-35S following digestion with the restriction enzyme SalI and cloned into the corresponding restriction site of the binary vector pZS199 to generate plasmid pSH24. Vector pZS199 was described in detail in WO 00/11176. The vector contains right and left T-DNA borders for integration of the expression cassette into the host plant genome and a neomycin phosphotransferase II gene linked to a cauliflower mosaic virus 35S promoter, which confers kanamycin selection for transgenic plant cells.

Plasmid pSH24, which contains a fusion of the barley putative HGGT cDNA with the cauliflower mosaic virus 35S promoter and the nos termination sequences in vector pZS199, was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Cultures derived from these cells were used for transformation of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks according to the protocol described by Rogers, S. G., Horsch, R. B., and Fraley, R. T. (1986) *Methods Enzymol.* 118:627–648. Tobacco leaf disks were also transformed with *A. tumefaciens* harboring only the pZS199 vector. Transformed tobacco callus was selected by the ability of cells to grow on media containing kanamycin at a concentration of 300 mg/L. In addition, expression of the transgene in these cells was confirmed by Northern blot analysis using a radiolabeled probe derived from the full-length barley putative HGGT cDNA (bdl2c.pk006.o2 cDNA; SEQ ID NO: 1).

Transformed callus arising from leaf disks was analyzed for tocotrienol production following ten to twelve days of growth on fresh kanamycin-containing media. Analyses were conducted using 10 to 50 mg (dry weight) of lyophilized tobacco callus. The transgenic tissue was initially homogenized in 3 mL of methanol:chloroform (2:1 v/v) in a 13×100 mm glass test tube. Following 2 h of incubation, 1 mL of chloroform and 1.8 mL of water were added. The organic and aqueous layers were thoroughly mixed and then partitioned by centrifugation. The organic layer was recovered, dried under nitrogen, and resuspended in 175 μL of heptane. The tocopherol and tocotrienol content of the organic extract was then determined using HPLC. Resolution of these compounds was achieved using a Hewlett Packard LiChroSpher Si 60 column (25 cm length; 5μ particle size) and a solvent system consisting of heptane: isopropanol (99:1 v/v) with a flow rate of 1 mL/min. Analytes were detected and quantified by fluorescence with excitation at 292 nm and emission at 335 nm. Tocopherol and tocotrienol molecular species were identified by mobility relative to standard compounds and quantified relative to δ-tocopherol, which was added as an internal standard. Using this methodology, tobacco callus expressing the barley putative HGGT cDNA was found to contain the following tocotrienol species: α-, β, γ- and δ-tocotrienol. In contrast, no tocotrienols were detected in tobacco callus transformed with only the pZS199 expression vector. In these studies, expression of the barley HGGT cDNA was mediated by the cauliflower mosaic virus 35S promoter. The analyses were conducted using an equivalent amount of tobacco callus.

This result thus demonstrates that the barley bdl2c.pk006.o2 cDNA disclosed in SEQ ID NO: 1 encodes a functional homogentisate geranylgeranyl transferase (HGGT) whose expression in transgenic plant cells results in tocotrienol production. Amounts of tocotrienol in tobacco callus expressing the barley HGGT cDNA were as high as 164.7-mg/kg dry weight, and the total tocopherol and tocotrienol content of these cells was as much as ten-fold higher than that in cells transformed with only the pZS199 vector (Table 2).

TABLE 2

Total tocopherol and tocotrienol content of tobacco callus transformed with the binary vector pZS199 (Vector Control) or with the barley HGGT cDNA operably linked to the cauliflower mosaic virus 35S promoter in pZS199 (+Barley HGGT cDNA). (The values shown were obtained from independent transgenic events.)

Transgenic Events	Total Tocopherol Content (mg/kg dry weight)	Total Tocotrienol Content (mg/kg dry weight)
Vector Control		
Event 1	17.5	n.d.*
Event 2	16.4	n.d.
Event 3	12.8	n.d.
Event 4	14.6	n.d.
+Barley HGGT cDNA		
Event 1	11.8	161.1
Event 2	14.0	155.4
Event 3	12.3	118.5
Event 4	14.3	164.7
Event 5	9.7	121.1

*n.d., not detected.

To confirm the identity of tocotrienols in transgenic tobacco callus expressing the barley HGGT cDNA, the organic extract from these cells was analyzed by gas chromatography-mass spectrometry. These analyses were performed using a Hewlett Packard 6890 gas chromatograph interfaced with a Hewlett Packard 5973 mass selective detector (MSD). Samples were separated with a 15-m×0.25-mm (inner diameter) DB-1HT column (J&W Scientific). The oven temperature was programmed from 125° C. (4-min hold) to 240° C. (12-min hold) at a rate of 10°

C./min. The ionization potential of the MSD was 70 eV. Using these conditions, compounds were detected with mass spectra identical to those of tocotrienol standards. The mass spectra of α- and γ-tocotrienol that were detected in extracts of tobacco callus expressing the barley HGGT cDNA contained molecular ions for α-tocotrienol (m/z 424) and γ-tocotrienol (m/z 410) as well as M^+-219 ions arising from loss of the side chain and M^+-259 ions arising from cleavage of the chroman ring and accompanying rearrangement as described (Nair and Zenaida (1968) *Arch. Biochem. Biophys.* 127:413–418).

These results conclusively demonstrate the ability to produce tocotrienols and to increase tocol content in transgenic plant cells by overexpression of the barley HGGT cDNA.

EXAMPLE 3

Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Wheat Seed

Wheat seeds and derivatives such as wheat bran and kernel are enriched in tocotrienols (Shin, T. S. (1994) *J. Chromatogr.* A 678:49–58) and thus represent potential sources of an HGGT cDNA. Homology searches were conducted using the nucleotide sequence of the barley HGGT cDNA (SEQ ID NO: 1) and expressed sequence tags (ESTs) generated from developing wheat kernel. These searches resulted in the identification of wheat EST wdk2c.pk012.f2:fis (SEQ ID NO: 34) that shared 94% identity with the barley HGGT cDNA (SEQ ID NO:1) over a span of 321 nucleotides. This high degree of sequence identity suggested that EST wdk2c.pk012.f2:fis (SEQ ID NO: 34) encodes an HGGT polypeptide. Based on sequence comparisons with the cDNA for barley HGGT (SEQ ID NO: 1), the cDNA corresponding to EST wdk2c.pk012.f2:fis (SEQ ID NO: 34) lacked coding sequence for at least 200 N-terminal amino acids. Functional characterization of the wheat HGGT-like polypeptide corresponding to EST wdk2c.pk012.f2:fis (SEQ ID NO: 34) thus required isolation of additional 5' coding sequence.

Nested PCR was conducted to isolate a full-length cDNA for the wheat HGGT-like polypeptide. The template for the initial amplification reaction consisted of 25 ng of a mixture of cDNA libraries prepared from developing kernels, roots and seedlings of wheat. The libraries consisted of cDNA inserts cloned into the plasmid pBluescript SK(+). The sense oligonucleotide primer for this reaction corresponded to sequence in pBluescript SK(+) that flanks the 5' end of cDNA inserts, and the antisense primer corresponded to sequence in the EST wdk2c.pk012.f2:fis (SEQ ID NO: 34). The sequences of the corresponding primers were as follows: 5'-GCCAAGCTCGGAATTAACCCTCA-3' (sense) (SEQ ID NO: 35) and 5'-CACAGTACAAGGAAAATCCMGCA-3' (antisense) (SEQ ID NO: 36). The reaction was conducted in a volume of 20 μL, and amplification was achieved using Advantage cDNA polymerase mix. The initial PCR cycling conditions were as follows: denaturation at 94° C. for 30 s, annealing at 68° C. for 30 s, and extension at 72° C. for 4 min. The annealing temperature was then lowered by 0.5° C. for each of the subsequent 9 cycles. This was then followed by 25 cycles with an annealing temperature of 63° C. The unpurified product of this reaction was then diluted 200-fold and used as template in a reaction that was conducted using the same amplification conditions as in the first reaction. The respective sense and antisense primers for this reaction corresponded to sequences in pBluescript SK(+) and EST wd2kc.pk012.f2:fis (SEQ ID NO: 34) that were flanked by the primers from the first reaction. The sequences of these "nested" oligonucleotide primers used in the second reaction were as follows: 5'-GCCGCTCTAGAACTAGTGGATCCCC-3' (sense) (SEQ ID NO: 37) and 5'-TCCAAGCATTGGATAGGGTATCA-3' (antisense) (SEQ ID NO: 38). The product of this second PCR reaction was subcloned into the vector pGEM-T Easy (Promega) according to the manufacturer's protocol, and complete DNA sequence was subsequently obtained from the subcloned PCR products. Using this methodology, full-length coding sequence was obtained for a 408 amino acid polypeptide that shared 86.7% identity with the barley HGGT. The sequences of the full-length cDNA (designated "wdk2c.pk012.f2:cgs") from wheat and the corresponding polypeptide are disclosed in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

In order to establish the function of the wheat HGGT-like polypeptide in SEQ ID NO: 4, the corresponding wdk2c.pk012.f2:cgs cDNA (SEQ ID NO: 3) was operably linked to a cauliflower mosaic virus 35S promoter and expressed in tobacco callus.

The complete open-reading frame of the wheat cDNA was initially amplified by PCR with oligonucleotides that contained added restriction enzyme sites to facilitate cloning into the plant expression vector. The oligonucleotide primers used for the PCR reaction were as follows: 5'-ttggaattcGTGGCCGCCCGGCGAGGATGC-3' (sense) (SEQ ID NO: 39) and 5'-ttaccTCACATCTGCTGGCCCTTGTAC-3' (antisense) (SEQ ID NO: 40). (Note: The bases in lower case contain the added restriction sites, which are underlined, and flanking sequence to facilitate restriction enzyme digestion.) The template for the reaction was the wheat HGGT-like cDNA (wdk2c.pk012.f2:cgs; SEQ ID NO: 3) cloned in PGEM-T Easy, and amplification was achieved using Pfu polymerase (Stratgene). The product of this reaction was purified by agarose gel electrophoresis, digested with EcoRI and KpnI, and cloned into the corresponding sites of the plant expression vector pML135 to generate the plasmid pWhtHGGT-35S. The vector pML135 was generated from vector pML63, which has been described previously in WO 00/11176 (the contents of which are hereby incorporated by reference), by replacement of the beta-glucuronidase (GUS) gene with a polylinker that contained the restriction enzyme recognition sites for PmlI, EcoRI, SmaI, and KpnI. pWhtHGGT-35S contains the open-reading frame of the wheat HGGT-like cDNA (wdk2c.pk012.f2:cgs; SEQ ID NO: 3) flanked on its 5'-end by the cauliflower mosaic virus 35S promoter and on its 3'-end by transcriptional termination sequence for the nopaline synthase gene. This expression cassette was subsequently moved as a SalI fragment from pWhtHGGT-35S and cloned into the corresponding site of the binary vector pZS199 (described in Example 2) to generate the plasmid pEC17. *Agrobacterium tumefaciens* LBA4404 cells were subsequently transformed as described in Example 2. The transformed *A. tumefaciens* cells were then used for infection of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks using protocols detailed in Example 2.

The resulting tobacco callus transformed with the wheat HGGT-like cDNA (wdk2c.pk012.f2:cgs; SEQ ID NO: 3) operably linked to the cauliflower mosaic virus 35S promoter was maintained and analyzed for tocopherol and tocotrienol content as described in Example 2. Tobacco callus expressing the wheat HGGT cDNA accumulated molecular species of tocotrienols including α-, β-, γ- and δ-tocotrienol. Of five independent events analyzed, amounts

of tocotrienols detected were as high as 140 mg/kg (dry weight). In contrast, no tocotrienol accumulation was detected in tobacco callus transformed with the pZS199 binary vector lacking cDNA insert. The analyses were conducted using an equivalent amount of tobacco callus.

These results thus demonstrate that the wheat wdk2c.pk012.f2:cgs cDNA disclosed SEQ ID NO: 3 encodes a functional HGGT polypeptide whose expression in transgenic plant cells results in the production of tocotrienols.

EXAMPLE 4

Identification and Functional Characterization of a Homogentisate Geranylgeranyl Transferase (HGGT) cDNA from Rice Seed

Rice seeds and byproducts such as rice bran are enriched in tocotrienols (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129–131) and are thus potential sources of homogentisate geranylgeranyl transferase (HGGT) cDNAs. Using methodology similar to that described in Example 2, isolation of a HGGT cDNA from rice seed was achieved through a PCR strategy that employed degenerate oligonucleotide primers designed from partially conserved domains in homogentisate phytyltransferases (HPTs) disclosed in WO 00/68393. The term "degenerate oligonucleotide" refers to a synthesized mixture of a nucleotide sequence in which a given position within the sequence can be represented by more than one nucleotide in the mixture. The template for PCR amplification reactions was a cDNA library prepared from developing rice seeds harvested at two to five days after pollination. The protocols for RNA isolation from rice seeds and synthesis of cDNA inserts were the same as those used in the preparation of a barley developing seed cDNA library described in Example 2. The resulting cDNA inserts were cloned in a 5'→3' orientation into the EcoRI and XhoI sites of the Lambda Uni-ZAP XR phage vector (Stratagene). The vector containing the developing rice seed cDNA inserts was then packaged and subsequently amplified according to the manufacturer's protocol (Stratagene).

The resulting rice developing seed cDNA library was used as template for PCR amplification at a total amount of 5×10^6 plaque-forming units in a 50-μL reaction volume. The degenerate oligonucleotides HPT5' (SEQ ID NO: 20) and HPT3' (SEQ ID NO: 22) (as described in Example 2) were used as the sense and antisense primers, respectively. These primers were included in the reaction at a final concentration of 1.5 μM. Amplification was achieved using Advantage cDNA polymerase mix (Clontech). Forty amplification cycles were conducted using the following temperatures and times for each cycle: 94° C. for 1 min, 50° C. for 1 min, and 72° C. for 1.25 min. The unpurified product of this reaction was diluted seven-fold, and 1 uL of this dilution was used as template in a second PCR reaction. This reaction was conducted using the same reaction conditions as above. HPT3' (SEQ ID NO: 22) was again used as the antisense primer, and HPT5' (SEQ ID NO: 20) was replaced as the sense primer with the degenerate oligonucleotide HPT5'-2 (SEQ ID NO: 41), which contained the following sequence: 5'-ATHGAYAARRTNAAYAARCC-3' (SEQ ID NO: 41). This oligonucleotide corresponds to the amino acid sequence IDK(I/VM)NKP (SEQ ID NO: 42), which is a partially conserved domain in HPT sequences disclosed in WO 00/68393. The coding sequence of this domain corresponds to a region upstream of the HPT5' sequence in HPT cDNAs. Using the HPT5'-2 (SEQ ID NO: 41) and HPT3' primers (SEQ ID NO: 22), products of approximately 700 bp were obtained from the second PCR reaction. The products were then purified and subcloned into vector PGEM-T Easy (Promega) according to the manufacturer's protocol. Upon partial sequencing, one of the PCR products was found to encode a polypeptide that was more related to HGGTs from barley and wheat than to HPTs disclosed in WO 00/68393.

Functional characterization of this putative rice HGGT required isolation of additional 5' and 3' sequence for the complete N'- and C'-termini of this polypeptide. This was achieved through a series of nested PCR reactions in which the rice developing seed cDNA library (described above) was used as the template. For all PCR reactions described below, forty amplification cycles were conducted using Advantage cDNA polymerase mix (Clontech) and the temperatures and times for each cycle were as follows: 94° C. for 1 min, 53° C. for 1 min, and 72° C. for 1.25 min. For isolation of the coding sequence of the complete C' terminus, sense oligonucleotide primers were designed based on the sequence of the PCR product encoding the partial rice HGGT-like polypeptide, and antisense primers were designed based on sequences in the Lambda Uni-Zap cDNA library vector. A PCR reaction was initially conducted that contained an aliquot of the rice developing seed cDNA library (5×10^6 plaque-forming units) and the following sense and antisense primers: 5'-GGAAGTGCATACTCT-GTTGATG-3' (SEQ ID NO: 43) and 5'GTAAAACGACG-GCCAGT-3' (M13–20 primer) (SEQ ID NO: 29). The unpurified products of this reaction were diluted 10-fold, and 1 μL of this dilution was used as template in a second reaction that contained the following nested sense and antisense primers: 5'-CTTGTATACTATTTGTAAGAGC-3' (SEQ ID NO: 44) and 5'-GTAATACGACTCACTAT-AGGGC-3' (T7 primer) (SEQ ID NO: 31). The product of this reaction was purified by agarose gel electrophoresis and subcloned into the vector pGEM-T Easy (Promega) using the method described by the manufacturer. Nucleotide sequence was then obtained for the entire subcloned PCR product. Comparison of the resulting sequence with those of barley and wheat HGGTs indicated that the PCR product encoded the complete C'-terminus of an HGGT-like polypeptide. For isolation of the coding sequence of the complete N'-terminus of the rice HGGT-like polypeptide, sense primers were designed based on sequences in the Lambda Uni-Zap cDNA library vector. The design of antisense primers was based on the coding sequence obtained for the C'-terminus of the rice HGGT-like polypeptide. A PCR reaction was initially conducted that contained an aliquot of the rice developing seed cDNA library (5×10^6 plaque-forming units) and the following sense and antisense primers: 5'-AACAGCTATGACCATG-3' (M13 reverse) (SEQ ID NO: 45) and 5'-ATAATTGCTCATGTGCATG-GTC-3' (SEQ ID NO: 46). The unpurified products of this reaction were diluted 10-fold, and 1 μL of this dilution was used as template in a second reaction that contained the following nested sense and antisense primers: 5'-AAAT-TAACCCTCACTAAAGGG-3' (modified T3 primer) (SEQ ID NO: 24) and 5'-CATGTAAATGATGTGATCCAC-3' (SEQ ID NO: 47). The product of this reaction was purified by agarose gel electrophoresis and subcloned into the vector pGEM-T Easy (Promega) according to the manufacturer's protocol. Results of sequence analysis indicated that the PCR product contained the coding region for the complete N'-terminus of an HGGT-like polypeptide (relative to barley and wheat HGGTs described in Examples 2 and 3).

Using the sequence information obtained above, the complete open-reading frame for the rice HGGT-like polypeptide was then amplified by PCR from the rice developing seed cDNA library. The oligonucleotide primers used in this reaction corresponded to sequences that flanked the open-reading frame of the rice HGGT-like polypeptide. These primers contained the following sequences: 5'-ttgcggccgcA-GACGATGCAAGCCTCATCGG-3' (sense) (SEQ ID NO: 48) and 5'-ttgcggccgcCTTGCCCTTGTGTATATAGTGC-3' (antisense) (SEQ ID NO. 49).(Note: The lower case, underlined sequence corresponds to an added NotI restriction site. The remaining lower case sequence was added to facilitate restriction enzyme digestion.) PCR amplification was conducted using Advantage cDNA polymerase mix and an aliquot of the rice developing seed cDNA library (3.5×10^6 plaque-forming units) in a 50 μL reaction volume. Thirty-five amplification cycles were performed using an annealing temperature of 57° C. The resulting cDNA product was purified by agarose electrophoresis and sub-cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's protocol. The sequence obtained from this cDNA (designated "rds1c.pk007.m9") is disclosed in SEQ ID NO: 5, and the deduced amino acid sequence is disclosed in SEQ ID NO: 6. The polypeptide encoded by the PCR product shared 68.6% amino acid sequence identity with the barley HGGT (SEQ ID NO: 2) and 66.3% identity with the wheat HGGT (SEQ ID NO: 4).

Functional characterization of the putative rice HGGT polypeptide (SEQ ID NO: 6) was conducted by transgenic expression in tobacco callus. For these studies, the coding sequence of the putative rice HGGT (SEQ ID NO: 6) was operably linked to the cauliflower mosaic virus 35S promoter. To facilitate cloning into the plant expression vector, the coding sequence of the putative rice HGGT was amplified with primers that contained a flanking SmaI and KpnI restriction sites, which are underlined in the sequences below. The sequences of the oligonucleotide primers used in this reaction were as follows: 5'-TTCCCGGGAGACGAT-GCAAGCCTCATCG-3' (sense) (SEQ ID NO: 50) and 5'-TTGGTACCGTGTATATAGTGCTCACTGCAC-3' (antisense) (SEQ ID NO: 51). PCR amplification was conducted with Pfu polymerase, and the rice putative HGGT cDNA (rds1c.pk007.m9; SEQ ID NO: 5 was used as the template. The product of this reaction was purified by agarose gel electrophoresis, digested with SmaI and KpnI, and then cloned into the corresponding restriction sites of the plant expression vector pML135 (which is described in Example 3). The resulting plasmid pRiceHGGT-35S contained an expression cassette consisting of the putative rice HGGT open reading frame flanked on its 5'-end by the cauliflower mosaic virus 35S promoter and on its 3'-end by transcriptional termination sequence for the nopaline synthase (nos) gene. This expression cassette was then inserted as a Sa/I restriction fragment into the corresponding site of the binary vector pZS199 (which is described in WO 00/11176 (the contents of which are hereby incorporated by reference) and in Examples 2 and 3) to generate plasmid pEC1 8. *Agrobacterium tumefaciens* LBA4404 cells were subsequently transformed with pEC18 as described in Example 2. The transformed *A. tumefaciens* cells were then used for infection of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks using protocols detailed in Example 2.

The resulting tobacco callus transformed with the putative rice HGGT cDNA (rds1c.pk007.m9; SEQ ID NO: 5) operably linked to the cauliflower mosaic virus 35S promoter was maintained and analyzed for tocopherol and tocotrienol content as described in Example 2. Tobacco callus expressing the putative rice HGGT cDNA accumulated molecular species of tocotrienols including α-, β-, γ- and δ-tocotrienol. Of four independent transformation events analyzed, amounts of tocotrienols detected were as high as 95 mg/kg (dry weight). In contrast, no tocotrienol accumulation was detected in tobacco callus transformed with the pZS199 binary vector lacking cDNA insert.

These results thus demonstrate that the rice rds1c.pk007.m9 cDNA disclosed in SEQ ID NO:5 encodes a functional HGGT polypeptide whose expression in transgenic plant cells is sufficient for the production of tocotrienols.

EXAMPLE 5

Production of Tocotrienols in Somatic Soybean Embryos

Somatic soybean embryos have been used as model for the prediction of transgenic phentoypes in soybean seeds (Kinney, A. J. (1996) *J. Food Lipids* 3:273–292). Somatic soybean embryos and seeds are enriched in tocopherols, but contain little or no tocotrienols (Coughlan, unpublished result; The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129–131). To demonstrate the ability to produce tocotrienols in somatic soybean embryos, the barley HGGT cDNA (bdl2c.pk006.o2; SEQ ID NO: 1) was expressed in this tissue under control of a strong seed specific promoter. The open-reading frame of bdl2c.pk006.o2 was initially amplified by PCR to generate flanking NotI sites for cloning into the soybean expression vector. The sequences of the sense and antisense oligonucleotide primers used in this reaction were as follows: 5'-ttgcggccgcAGGATGCAAGCCGT-CACGGCGGCAGCCG-3' (SEQ ID NO: 52) and 5'-ttgcg-gccgcTTCACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 53). (Note: The lower case, underlined nucleotide sequences correspond to added NotI restriction sites.) PCR amplification was achieved using Pfu polymerase, and an aliquot of the barley developing seed cDNA library described in Example 2 was used as the template. The product of this PCR reaction was purified by agarose gel electrophoresis and subcloned into pCR-Script-AMP (Stratagene) as described in the manufacturer's protocol. The amplified open-reading frame of the barley HGGT was then released as a NotI fragment and cloned into the corresponding site of soybean expression vector pKS121 to generate plasmid pSH13. The construction of vector pKS121 was previously described in WO 02/00904 (the contents of which are hereby incorporated by reference). This vector contains the seed specific promoter for the Kunitz trypsin inhibitor-3 (Kti3) gene (Jofuku and Goldberg (1989) *Plant Cell* 1:1079–1093) linked via a NotI restriction site to the 3' transcriptional termination sequence of the Kti3 gene. Bacterial selection for the pKS121 plasmid is conferred by a hygromycin B phosphotransferase gene (Gritz and Davies (1983) *Gene* 25:179–188) under control of the promoter for the T7 RNA polymerase promoter. Plasmid pSH13 thus contains a soybean expression cassette consisting of the barley HGGT open-reading frame operably linked on its 5' end to the Kti3 promoter and on its 3' end to the Kti3 transcription termination sequence.

Somatic soybean embryos were transformed with pSH13 using the biolistic method. For these experiments, pSH13 was co-bombarded with the plasmid pKS18HH at a 10:1 molar ratio of the two plasmids. Plasmid pKS18HH (U.S. Pat. No. 5,846,784 (the contents of which are hereby incorporated by reference)) contains hygromycin B phospho-

transferase gene under control of the cauliflower mosaic 35S promoter, which allows for selection of transformed plant cells by resistance to the antibiotic hygromycin B. The protocol used for transformation of somatic soybean embryos is described below.

To induce somatic embryos, cotyledons, 3–5 mm in length dissected from surface sterilized, immature seeds of a soybean cultivar Jack were cultured in the light or dark at 26° C. on an appropriate agar medium for 6–10 weeks. Somatic embryos that produce secondary embryos were then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular staged embryos, the suspensions were maintained as described below.

Soybean embryogenic suspension cultures were maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26° C. with florescent lights on a 16:8 hour day/night schedule. Cultures were subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures were then co-transformed with pSH13 and pKS18HH by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70; U.S. Pat. No. 4,945,050). A DuPont Biolisticä PDS1000/HE instrument (helium retrofit) was used for these transformations.

To 50 mL of a 60 mg/mL 1 mm gold particle suspension were added (in order): 5 mL DNA (1 mg/mL), 20 mL spermidine (0.1 M), and 50 mL $CaCl_2$ (2.5 M). The particle preparation was then agitated for three minutes, spun in a microfuge for ten seconds and the supernatant removed. The DNA-coated particles were then washed once in 400 mL 70% ethanol and resuspended in 40 mL of anhydrous ethanol. The DNA/particle suspension was sonicated three times for one second each. Five mL of the DNA-coated gold particles was then loaded on each macro carrier disk.

Approximately 300–400 mg of a two-week-old suspension culture was placed in an empty 60×15-mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately five to ten plates of tissue were bombarded. Membrane rupture pressure was set at 1100 psi and the chamber was evacuated to a vacuum of 28 inches mercury. The tissue was placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue was divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media was exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media was refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue was removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line was treated as an independent transformation event. These suspensions were then subcultured and maintained as clusters of immature embryos. Immature embryos at this stage produce storage products, including storage lipids that are similar in composition to zygotic embryos at a similar stage of development (see WO 94/11516 (the contents of which are hereby incorporated by reference)).

The resulting somatic embryos transformed with the barley HGGT expression cassette from pSH13 were examined for tocotrienol production using extraction and analytical techniques described in Example 2. In at least three of 19 transgenic events analyzed, the accumulation of α, γ, and δ molecular species of tocotrienol was detected. In contrast, no tocotrienols were detected in untransformed somatic soybean embryos.

These results thus demonstrate the ability to produce tocotrienols in a crop plant that does not normally accumulate these antioxidant molecules through the transgenic expression of a nucleic acid fragment encoding an HGGT polypeptide.

EXAMPLE 6

Production of Tocotrienols in Corn (*Zea mays*) Seed

Corn oil, which is derived primarily from the embryo of corn seeds, is typically enriched in tocopherols but contains little or no tocotrienols (The Lipid Handbook, 2nd Edition, Gunstone, F. D., et al., Eds., Chapman and Hall, London, 1994, pp. 129–131). Based on results disclosed in Examples 2 and 5, the barley HGGT cDNA (SEQ ID NO: 1) can be expressed in seed embryo of corn to produce tocotrienols and to increase the tocol antioxidant content of this tissue and the extracted oil. As described below, this can be achieved by transforming corn with the barley HGGT open reading frame operably linked on its 5' end to an embryo specific promoter, such as the promoter for the corn 16 kDa oleosin gene (Lee, K. and Huang, A. H. (1994) *Plant Mol. Biol.* 26:1981–1987).

Initially, the open reading frame of the barley HGGT cDNA (SEQ ID NO: 1) was amplified by PCR to generate flanking PacI restriction sites, which allow for the cDNA to be cloned into a corn embryo expression cassette. The sense and antisense oligonucleotide primers used for PCR amplification were as follows: 5'-atttaattaaGCCGGCGAGGATGCAAGCCGTC-3' (SEQ ID NO: 54) and 5'-tattaattaaTTCACATCTGCTGGCCCTTGTAC-3' (SEQ ID NO: 55). (The lower case, underlined nucleotides correspond to the added PacI sites, and the additional lower case nucleotides are added to facilitate restriction enzyme digestion.) Amplification can be achieved using Pfu polymerase (Stratagene) and reaction conditions similar to those described in Example 5. The resulting PCR product derived from the barley HGGT cDNA was purified, digested with PacI, and then cloned into the corresponding site of the corn embryo expression vector pTG10 to generate plasmid pKR242. Vector pTG10 contains a corn expression cassette consisting of 959 base pairs of the promoter for the corn 16 kDa oleosin gene which has been previously described in WO 99/64579 (the contents of which are hereby incorporated by reference). This promoter element is linked via a PacI restriction site to 330 base pairs of the 3' transcription termination sequence of the corn 16 kDa oleosin gene (Lee, K. and Huang, A. H. (1994) *Plant Mol. Biol.* 26:1981–1987). Bacterial selection in pTG10 is conferred by a hygromycin B phosphotransferase gene (Gritz, L. and Davies, J. (1983) *Gene* 25:179–188) under control of the promoter for the T7 RNA polymerase gene. Plasmid pKR242 thus contains an expression cassette composed of the barley HGGT open reading frame operably linked on its 5' end to the promoter for the corn 16 kDa oleosin gene and on its 3' end to the transcription termination sequence of the corn 16 kDa oleosin gene. The entire expression cassette is flanked by AscI restriction sites. Following digestion with AscI, the expression cassette of pKR242 was removed and inserted into the corresponding site of the binary vector PHP15578 to generate plasmid PHP18749. The binary vector PHP15578 has been previ-

ously described in WO 02/00904 (the contents of which are hereby incorporated by reference). PHP15578 contains right and left border regions to facilitate Agrobacterium-mediated transformation of corn, and plant selection is conferred by a cauliflower mosaic virus 35S promoter-bialaphos selectabe marker element.

Plasmid PHP18749 can be used for the generation of transgenic corn that expresses the barley HGGT cDNA in an embryo specific manner. An *Agrobacterium*-based protocol can be used for the transformation of corn with expression elements from PHP18749 as described below.

Transformation of Corn Mediated by *Agrobacterium:*

Freshly isolated immature embryos of corn, about 10 days after pollination (DAP), can be incubated with the *Agrobacterium*. The preferred genotype for transformation is the highly transformable genotype Hi-II (Armstrong (1991) *Maize Gen. Coop. Newsletter* 65:92–93). An F, hybrid created by crossing a Hi-II with an elite inbred may also be used. After *Agrobacterium* treatment of immature embryos, the embryos can be cultured on medium containing toxic levels of herbicide. Only those cells that receive the herbicide resistance gene, and the linked gene(s), grow on selective medium. Transgenic events so selected can be propagated and regenerated to whole plants, produce seed, and transmit transgenes to progeny.

Preparation of *Agrobacterium:*

The engineered *Agrobacterium tumefaciens* LBA4404 can be constructed to contain plasmid PHP18749, as disclosed in U.S. Pat. No. 5,591,616 (the contents of which are hereby incorporated by reference). To use the engineered construct in plant transformation, a master plate of a single bacterial colony transformed with PHP18749 can be prepared by inoculating the bacteria on minimal AB medium and allowing incubation at 28° C. for approximately three days. (The composition and preparation of minimal AB medium has been previously described in WO 02/00904 (the contents of which are hereby incorporated by reference.) A working plate can then be prepared by streaking the transformed *Agrobacterium* on YP medium (0.5% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) sodium chloride, 1.5% (w/v) agar) that contains 50 μg/mL of spectinomycin.

The PHP18749-transformed *Agrobacterium* for plant transfection and co-cultivation can then be prepared one day prior to corn transformation. Into 30 mL of minimal A medium (prepared as described in Application WO 02/009040) in a flask was placed 50 μg/mL spectinomycin, 100 μM acetosyringone, and about a ⅛ loopful of *Agrobacterium* from a one to two-day-old working plate. The *Agrobacterium* can then be grown at 28° C. with shaking at 200 rpm for approximately fourteen hours. At mid-log phase, the *Agrobacterium* can be harvested and resuspended at a density of 3 to 5×108 CFU/mL in 561Q medium that contains 100 μM acetosyringone using standard microbial techniques. The composition and preparation of 561Q medium was described in WO 02/009040.

Immature Embryo Preparation:

Nine to ten days after controlled pollination of a corn plant, developing immature embryos are opaque and 1–1.5 mm long. This length is the optimal size for infection with the PHP18749-transformed *Agrobacterium*. The husked ears can be sterilized in 50% commercial bleach and one drop Tween-20 for thirty minutes, and then rinsed twice with sterile water. The immature embryos can then be aseptically removed from the caryopsis and placed into 2 mL of sterile holding solution consisting of medium 561 Q that contains 100μM of acetosyringone.

Agrobacterium Infection and Co-cultivation of Embryos:

The holding solution can be decanted from the excised immature embryos and replaced with the PHP18749-transformed *Agrobacterium*. Following gentle mixing and incubation for about five minutes, the *Agrobacterium* can be decanted from the immature embryos. Immature embryos were then moved to a plate of 562P medium, the composition of which has been previously described in WO 02/009040. The immature embryos can be placed on this media scutellum surface pointed upwards and then incubated at 20° C. for three days in darkness. This can be followed by incubation at 28° C. for three days in darkness on medium 562P that contains 100 μg/mL carbenecillin as described in U.S. Pat. No. 5,981, 840.

Selection of Transgenic Events:

Following incubation, the immature embryos can be transferred to 5630 medium, which can be prepared as described in WO 02/009040. This medium contains Bialaphos for selection of transgenic plant cells as conferred by the BAR gene that is linked to barley HGGT expression cassette. At ten to fourteen-day intervals, embryos were transferred to 5630 medium. Actively growing putative transgenic embryogenic tissue can be after six to eight weeks of incubation on the 5630 medium.

Regeneration of T_0 Plants:

Transgenic embryogenic tissue is transferred to 288W medium and incubated at 28° C. in darkness until somatic embryos matured, or about ten to eighteen days.

Individual matured somatic embryos with well-defined scutellum and coleoptile are transferred to 272 embryo germination medium and incubated at 28° C. in the light. After shoots and roots emerge, individual plants are potted in soil and hardened-off using typical horticultural methods.

288W medium contains the following ingredients: 950 mL of deionized water; 4.3 g of MS Salts (Gibco); 0.1 g of myo-inositol; 5 mL of MS Vitamins Stock Solution (Gibco); 1 mL of zeatin (5 mg/mL solution); 60 g sucrose; 8 g of agar (Sigma A-7049, Purified), 2 mL of indole acetic acid (0.5 mg/mL solution*); 1 mL of 0.1 mM ABA*; 3 mL of Bialaphos (1 mg/mL solution*); and 2 mL of carbenicillin (50 mg/mL solution). The pH of this solution is adjusted to pH 5.6. The solution is autoclaved and ingredients marked with an asterisk (*) are added after the media has cooled to 60° C. Medium 272 contains the following ingredients: 950 mL of deionized water; 4.3 g of MS salts (Gibco); 0.1 g of myo-inositol; 5 mL of MS vitamins stock solution (Gibco); 40 g of Sucrose; and 1.5 g of Gelrite. This solution is adjusted to pH 5.6 and then autoclaved.

Confirmation of Transformation:

Fifty putative transgenic events were recovered and initially confirmed using a leaf paint test with bialaphos herbicide. The subsequent lack of a herbicide-injury lesion indicated the presence and action of the herbicide selectable marker and the plants were self pollinated and taken through seed fill and maturation. More than 150 seeds were obtained from each of 48 of the initially selected, transformed lines. The germ from 10 seeds of each of the 48 lines and one non-transgenic control was partially removed by dissection, weighed and combined in a 1.5 ml micro-centrifuge tube. One ml of heptane, which also contained 80 μg of α-tocopherol acetate as internal standard, was added and the germ material was ground using a small plastic pestle. The ground germ material was further extracted during 2 hrs of agitation at room temperature. Total tocopherols and tocotrienols were analyzed on one aliquot of extract using the HPLC

separation and fluorescence detection procedure described in Example 2. The total fatty acid content of the heptane extract was determined by adding 2.5 mg of Tri-heptadecanoylglyceride to a 50 μl aliquot and forming the methyl esters by transesterification in 1 ml of methanol containing 0.25% sodium methoxide. The fatty acid methyl esters were extracted into 1 ml of heptane after addition of 1 ml of 1 M NaCl to the transesterification reaction. Total fatty acids were determined by separation of the methyl esters by GLC, detection by flame ionization and comparison of peak areas to the peak area of the methlyheptadecanoate derived from the internal standard. α-, β-, γ- and δ-Tocotrienol was detected in all of the transgenic lines but not in the germ extract of the non-transgenic control. Gamma-tocopherol was the most prominent tocopherol present and γ-tocotrienol was the most prominent tocotrienol in the transgenic events. The total tocopherol content of the transgenic lines on an oil basis was similar to that of the non-transgenic control and the total tocotrienol varied by event but reached levels up to 3 times the endogenous tocopherol content. The results for all the events analyzed are shown in Table 3 and demonstrate that the barley HGGT gene under the control of a promoter that is active in maize embryo tissue is capable of introducing the capability to synthesize and store tocotrienol. The total oil soluble anti-oxidant content can be increased by at least 3-fold by the expressed chimeric gene.

TABLE 3

Total tocopherol per mg of oil and total tocotrienol per mg of oil in the germ of 48 trangenic maize lines expressing the barley HGGT gene under control of the maize oleosin promoter and one wild type control. Individual events are ranked by total tocotrienol content.

EVENT#	TOTAL TOCOPHEROL (NG/MG OF OIL)	TOTAL TOCOTRIENOL (NG/MG OF OIL)
A1532.064.7.25.1	2997.5	8368.5
A1532.064.8.11.1	2858.9	6274.0
A1532.064.7.14.1	2990.3	5791.1
A1532.064.8.8.1	2720.3	5273.8
A1532.064.7.16.1	3018.5	5188.9
A1532.064.8.16.1	2068.4	5146.2
A1532.064.7.8.1	2701.9	5074.8
A1532.064.7.22.1	2306.6	4601.8
A1532.064.8.17.1	1993.4	4565.4
A1532.064.7.4.1	2996.7	4372.7
A1532.064.8.15.1	2610.3	4336.6
A1532.064.7.19.1	3663.3	4186.1
A1532.064.7.5.1	1946.2	4104.0
A1532.064.7.23.1	2554.5	3914.6
A1532.064.8.20.1	2359.2	3913.3
A1532.064.8.14.1	2680.9	3728.6
A1532.064.7.20.1	2026.7	3681.1
A1532.064.8.24.1	2330.7	3679.9
A1532.064.7.18.1	2017.5	3621.9
A1532.064.7.1.1	2899.1	3477.0
A1532.064.8.22.1	2827.6	3333.6
A1532.064.7.3.1	4293.1	3248.2
A1532.064.8.7.1	3797.9	3093.1
A1532.064.7.15.1	2840.9	3091.1
A1532.064.8.9.1	3425.5	2391.8
A1532.064.8.18.1	2932.6	2391.6
A1532.064.7.7.1	2500.9	2327.0
A1532.064.8.12.1	2673.3	2315.3
A1532.064.7.24.1	3072.7	2089.2
A1532.064.8.13.1	2782.0	2062.6
A1532.064.7.12.1	2140.8	1824.4
A1532.064.7.11.1	3076.0	1805.6
A1532.064.8.3.1	1515.9	1770.7
A1532.064.7.10.1	2928.5	1692.5
A1532.064.7.17.1	4019.4	818.5
A1532.064.7.13.1	4047.7	775.1
A1532.064.8.2.1	2869.2	683.7

TABLE 3-continued

Total tocopherol per mg of oil and total tocotrienol per mg of oil in the germ of 48 trangenic maize lines expressing the barley HGGT gene under control of the maize oleosin promoter and one wild type control. Individual events are ranked by total tocotrienol content.

EVENT#	TOTAL TOCOPHEROL (NG/MG OF OIL)	TOTAL TOCOTRIENOL (NG/MG OF OIL)
A1532.064.8.1.1	2667.5	601.8
A1532.064.8.25.1	3068.6	481.5
A1532.064.7.21.1	3887.7	375.8
A1532.064.7.9.1	2320.7	357.8
A1532.064.8.5.1	2797.1	81.9
A1532.064.7.2.1	1186.8	56.0
A1532.064.8.6.1	3094.8	41.6
WT	2732.0	0

Since the transgene in the seed population from a self pollinated initial transformant is expected to be genetically segregating for the presence of the transgene and its copy number, 15 single seeds from events were selected by their bulked, excised-germ, tocotrienol content. Single seeds were ground in a ball-impact single seed grinder and 100 mg of the resulting powder was weighed into 1.5 ml micro-centrifuge tubes and extracted using 1 ml of heptane and 5.4 μg of the α-tocopherol acetate internal standard as describe for the bulk germ. Unlike bulk germ, wild type maize whole seed contains the set of α, β, and γ tocotrienols due to their presence in the endosperm tissue. Extracted tocols were separated and quantified using the HPLC method described above and the results expressed as parts per million tocopherol, tocotrienol and the sum of the compound classes is shown in Table 4.

TABLE 4

Total tocopherols, total tocotrienols and the sum of the two expressed parts per million in grain. The results are from 15 single seeds for each of 7 transgenic events chosen to represent a sampling of all events based on their bulk germ analysis. The first entries listed as "B73" followed by a letter are single seeds from a wild type control. Entries within an event are ranked by their total tocotrienol content.

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
B73L 10/23	57.3	8.5	65.8
B73N 10/23	47.4	7.5	54.9
B73H 10/23	88.6	7.3	95.9
B73M 10/23	63.4	7.1	70.5
B73I 10/23	44.4	6.7	51.1
B73O 10/23	52.2	6.3	58.5
B73B 10/17	41.1	5.9	47.0
B73J 10/23	52.5	5.6	58.1
B73E 10/23	49.6	5.5	55.1
B73F 10/23	52.5	5.3	57.8
B73A 10/17	17.2	3.5	20.7
B73C 10/17	30.6	3.3	33.9
B73D 10/17	29.6	2.5	32.1
B73K 10/23	23.3	2.1	25.4
B73G 10/23	33.7	1.2	34.9
2I A1532.064.7.2	98.6	21.5	120.1
2J A1532.064.7.2	70.6	15.3	85.9
2L A1532.064.7.2	27.8	14.1	41.9
2H A1532.064.7.2	25.4	11.4	36.8
2B A1532.064.7.2	38.9	11.2	50.1
2C A1532.064.7.2	37.5	10.5	48.0
2M A1532.064.7.2	46.0	9.9	55.9
2N A1532.064.7.2	38.4	9.6	48.0

TABLE 4-continued

Total tocopherols, total tocotrienols and the sum of the two expressed parts per million in grain. The results are from 15 single seeds for each of 7 transgenic events chosen to represent a sampling of all events based on their bulk germ analysis. The first entries listed as "B73" followed by a letter are single seeds from a wild type control. Entries within an event are ranked by their total tocotrienol content.

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
2A A1532.064.7.2	29.5	9.5	39.0
2K A1532.064.7.2	38.8	9.0	47.8
2G A1532.064.7.2	43.2	7.1	50.3
2E A1532.064.7.2	23.4	6.8	30.2
2O A1532.064.7.2	14.7	5.8	20.5
2F A1532.064.7.2	83.9	5.4	89.3
2D A1532.064.7.2	49.1	4.8	53.9
31G A1532.064.8.6	140.3	21.3	161.6
31J A1532.064.8.6	87.5	20.0	107.5
31K A1532.064.8.6	58.4	18.0	76.4
31A A1532.064.8.6	94.8	16.3	111.1
31M A1532.064.8.6	84.3	15.0	99.3
31LA1532.064.8.6	63.3	13.0	76.3
31IA1532.064.8.6	43.3	10.4	53.7
31DA1532.064.8.6	31.1	9.3	40.4
31HA1532.064.8.6	30.9	8.6	39.5
31FA1532.064.8.6	42.4	8.0	50.4
31BA1532.064.8.6	73.5	7.5	81.0
31CA1532.064.8.6	61.3	6.3	67.6
31EA1532.064.8.6	30.7	5.9	36.6
15AA1532.064.7.15	70.8	191.9	262.7
15MA1532.064.7.15	58.5	155.9	214.4
15NA1532.064.7.15	72.2	135.1	207.3
15IA1532.064.7.15	77.1	126.8	203.9
15OA1532.064.7.15	78.4	120.1	198.5
15JA1532.064.7.15	45.7	104.6	150.3
15EA1532.064.7.15	45.6	92.8	138.4
15LA1532.064.7.15	40.9	89.7	130.6
15FA1532.064.7.15	52.5	82.9	135.4
15DA1532.064.7.15	35.7	78.2	113.9
15BA1532.064.7.15	37.9	69.8	107.7
15KA1532.064.7.15	27.6	58.5	86.1
15CA1532.064.7.15	26.9	52.0	78.9
15GA1532.064.7.15	30.5	10.5	41.0
15HA1532.064.7.15	17.7	7.3	25.0
25BA1532.064.7.25	29.2	295.9	325.1
25CA1532.064.7.25	54.6	258.1	312.7
25HA1532.064.7.25	34.5	258.0	292.5
25GA1532.064.7.25	77.3	246.7	324.0
25MA1532.064.7.25	52.7	235.6	288.3
25KA1532.064.7.25	72.9	226.2	299.1
25FA1532.064.7.25	66.7	221.0	287.7
25DA1532.064.7.25	40.0	196.9	236.9
25LA1532.064.7.25	60.0	195.9	255.9
25EA1532.064.7.25	39.9	181.8	221.7
25AA1532.064.7.25	51.7	176.6	228.3
25NA1532.064.7.25	61.3	133.0	194.3
25IA1532.064.7.25	39.7	114.5	154.2
25OA1532.064.7.25	32.9	60.8	93.7
25JA1532.064.7.25	109.1	13.7	122.8
36FA1532.064.8.11	55.0	293.3	348.3
36BA1532.064.8.11	62.1	285.5	347.6
36JA1532.064.8.11	91.1	249.3	340.4
36KA1532.064.8.11	87.5	242.1	329.6
36DA1532.064.8.11	63.8	195.4	259.2
36MA1532.064.8.11	60.2	185.7	245.9
36CA1532.064.8.11	142.9	163.9	306.8
36GA1532.064.8.11	79.3	161.8	241.1
36LA1532.064.8.11	138.2	152.4	290.6
36IA1532.064.8.11	85.7	147.0	232.7
36EA1532.064.8.11	79.1	147.0	226.1
36HA1532.064.8.11	75.9	143.7	219.6
36NA1532.064.8.11	64.0	137.2	201.2
36OA1532.064.8.11	78.5	129.1	207.6
36AA1532.064.8.11	110.5	128.1	238.6
41CA1532.064.8.16	77.1	307.0	384.1

TABLE 4-continued

Total tocopherols, total tocotrienols and the sum of the two expressed parts per million in grain. The results are from 15 single seeds for each of 7 transgenic events chosen to represent a sampling of all events based on their bulk germ analysis. The first entries listed as "B73" followed by a letter are single seeds from a wild type control. Entries within an event are ranked by their total tocotrienol content.

seed and event number	ppm tocopherol in grain	ppm tocotrienol in grain	ppm total toco in grain
41FA1532.064.8.16	45.3	235.5	280.8
41IA1532.064.8.16	127.0	228.0	355.0
41DA1532.064.8.16	63.2	209.0	272.2
41BA1532.064.8.16	51.4	202.6	254.0
41EA1532.064.8.16	122.5	195.4	317.9
41OA1532.064.8.16	91.4	189.6	281.0
41LA1532.064.8.16	63.5	188.6	252.1
41NA1532.064.8.16	57.4	188.4	245.8
41HA1532.064.8.16	125.0	167.5	292.5
41MA1532.064.8.16	88.2	163.3	251.5
41AA1532.064.8.16	64.3	147.9	212.2
41JA1532.064.8.16	59.2	135.8	195.0
41KA1532.064.8.16	67.2	107.1	174.3
41GA1532.064.8.16	120.4	102.4	222.8
42JA1532.064.8.17	112.5	460.7	573.2
42FA1532.064.8.17	99.9	373.2	473.1
42IA1532.064.8.17	24.2	213.8	238.0
42OA1532.064.8.17	57.2	201.3	258.5
42LA1532.064.8.17	65.7	196.5	262.2
42EA1532.064.8.17	27.4	193.5	220.9
42AA1532.064.8.17	51.6	181.1	232.7
42NA1532.064.8.17	67.8	157.9	225.7
42BA1532.064.8.17	26.6	157.2	183.8
42DA1532.064.8.17	51.2	149.5	200.7
42CA1532.064.8.17	64.3	122.7	187.0
42GA1532.064.8.17	26.2	109.4	135.6
42KA1532.064.8.17	98.8	105.6	204.4
42HA1532.064.8.17	38.8	104.6	143.4
42MA1532.064.8.17	54.4	19.4	73.8

The 15 wild type seeds had an average tocopherol content of 45.6 ppm, tocotrienol contents that ranged from 1.2 to 8.5 ppm and an averaged 5.2 ppm. Event number A15232.064.8.6 which was the lowest ranked transgenic had similar average values of 64.7 and 12.3 ppm for tocopherols and tocotrienols respectively. By contrast, event A1532.064.8.17 that was the top ranked event by bulk germ tocotrienol in oil had single seed tocotrienol values that ranged from 19.4 ppm to 460.7 ppm. The average tocopherol content in the event remained at 57.8 ppm. The transgene has no apparent effect on the ability of the maize seed to store tocopherols while it greatly increase the content of tocotrienol. In a population of segregating seeds, it is reasonable to expect at least one wild type seed in a sample of 15. If the lowest ranking seed in event A1532.064.8.17 is excluded from the tocotrienol average on that basis the average becomes 194.8 ppm and the total of tocopherol and tocotrienol is 252.8 ppm. Those values are conservative estimates of the maximum phenotype expected from the transgene in this event since the population is still segregating for transgene ploidy. If one assumes that the top 25% of seed by ranking is representative of the homozygous condition (assumes one segregating genetic locus) the average tocotrienol estimate is about 315 ppm. That value combined with the average tocopherol content gives a combined estimate of about 373 ppm.

Average expected values for total tocopherol and total tocotrienol in corn seeds is in the range 40 to 90 ppm (see Weber, E. J. 1987. Lipids of the Kernel. Page 335 in: Corn:

Chemistry and Technology. S. A. Watson and P. E. Ramstad, eds. American Association of Cereal Chemists, Inc. St. Paul, Minn.). The expression of the barley HGGT increases the tocotrienol levels from about 10 ppm to between 100 and 400 ppm and the total tocopherol+tocotrienol content to between 120 and 450 ppm.

EXAMPLE 7

Characterization of cDNA Clones Encoding Proteins Similar to *Arabidopsis thaliana* Homogentisate Phytyltransferase (Also Known as Tocopherol Polyprenyltransferase)

BLASTP searches using amino acid sequences deduced from clones listed in Table 3 revealed similarity of these polypeptides encoded by the cDNAs homogentisate phytyltransferase from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO: 13). Shown in Table 5 are the BLASTP results for amino acid sequences deduced from the entire cDNA inserts comprising the indicated clone ("FIS").

TABLE 5

BLAST Results for Sequences Encoding Polypeptides Homologous to HGGT

Clone	Status	BLAST pLog Score NCBI General Identifier No. 17104828 (SEQ ID NO: 13)
bdl2c.pk006.o2	FIS	94.00
cco1n.pk087.l17: fis	CGS	85.52
p0058.chpbj67r: fis	FIS	91.00
rds1c.pk007.m9	FIS	90.70
wdk2c.pk012.f2: cgs	CGS	95.00

The nucleotide sequence of clone bdl2c.pk006.o2 is shown in SEQ ID NO: 1. The amino acid sequence deduced from nucleotides 63 through 1286 of SEQ ID NO: 1 is shown in SEQ ID NO: 2. The nucleotide sequence of clone cco1n.pk087.117:cgs is shown in SEQ ID NO: 7. The amino acid sequence deduced from nucleotides 211 through 1353 of SEQ ID NO: 7 is shown in SEQ ID NO: 8. The nucleotide sequence of clone p0058.chpbj67r:fis is shown in SEQ ID NO: 9. The amino acid sequence deduced from nucleotides 357 through 1441 of SEQ ID NO: 9 is shown in SEQ ID NO: 10. The nucleotide sequence of clone rds1c.pk007.m9 is shown in SEQ ID NO: 5. The amino acid sequence deduced from nucleotides 6 through 1220 of SEQ ID NO: 5 is shown in SEQ ID NO :6. The nucleotide sequence of clone wdk2c.pk012.f2:cgs is shown in SEQ ID NO: 3. The amino acid sequence deduced from nucleotides 53 through 1279 of SEQ ID NO: 3 is shown in SEQ ID NO: 4.

The complete gene sequence (cgs) for clone cco1n.pk087.117 employed a PCR based amplification of the 5'-end of the transcript. Briefly, two nested primers (SEQ ID NOS: 67 and 68) were used in a RACE protocol (Frohman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:8998–9002) to generate fragments between a single point in the transcript and the 5' end. Primers oriented in the 5' direction (complementary to nucleotides 598–621 and 570–591 of SEQ ID NO: 7) were designed from the instant sequences, then commercially available RACE systems (BRL) were used to isolate specific 5' cDNA fragments (Ohara et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5673–5677; Loh et al. (1989) *Science* 243:217–220). Products generated by the RACE procedures can be combined to generate full-length cDNAs (Frohman and Martin (1989) *Techniques* 1:165). SEQ ID NO: 7 as listed is a cgs sequence and contains two adenine residues at positions 571 and 572. In contrast, the fis sequence variant of SEQ ID NO: 7 contains three adenine residues spanning positions 571 to 573 (positions corresponding to SEQ ID NO: 7). Primer SEQ ID NO: 67 was designed with three thymine residues in order to hybridize with the three adenine residues in the fis variant of SEQ ID NO: 7. The additional adenine residue in the fis variant of SEQ ID No: 7 would cause a frameshift to occur in the corresponding amino acid sequence.

Alignments of the amino acid sequences set forth in SEQ ID NOS: 2, 4, 6, 8 and 10 and the sequence from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO: 13) were performed. The data in Table 6 represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID Nos:2, 4, 6, 8 and 10 and the sequence from *Arabidopsis thaliana* (NCBI General Identification (GI) No. 17104828; SEQ ID NO:13).

TABLE 6

Percent Identity of Amino Acid Sequences Deduced from the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to HGGT

Clone	SEQ ID NO:	Percent Identity to NCBI General Identifier No. 17104828 (SEQ ID NO: 13)
bdl2c.pk006.o2	2	45.3
wdk2c.pk012.f2: cgs	4	45.5
rds1c.pk007.m9	6	44.8
cco1n.pk087.l17: cgs	8	42.5
p0058.chpbj67r: fis	10	46.5

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) CABIOS. 5:151–153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a HGGT.

EXAMPLE 8

Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the beta subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228–9238) can be used for expression of the instant polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites NcoI (which includes the ATG translation initiation codon), SmaI, KpnI and XbaI. The entire cassette is flanked by HindIII sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be

incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3–5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar Jack, can be cultured in the light or dark at 26° C. on an appropriate agar medium for 6–10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26° C. with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70–73, U.S. Pat. No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810–812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene* 25:179–188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 μL of a 60 mg/mL 1 μm gold particle suspension is added (in order): 5 μL DNA (1 μg/μL), 20 μL spermidine (0.1 M), and 50 μL $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μL 70% ethanol and resuspended in 40 μL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five μL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300–400 mg of a two-week-old suspension culture is placed in an empty 60×15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5–10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 9

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant HGGT polypeptides can be used to produce tocotrienols in microbes such as algal and cyanobacterial cells that contain an operable tocopherol biosynthetic pathway. Expression of cDNAs encoding the instant HGGT polypeptides in these cells are expected to result in the condensation of geranylgeranyl pyrophosphate and homogentisate. The product of the HGGT reaction 2-methyl-6-geranylgeranylbenzoquinol can then be converted to tocotrienols by tocopherol biosynthetic enzymes native to the host microbial cell. Tocotrienols can be produced in microbes by linking the cDNAs encoding the instant HGGT polypeptides with promoter elements that are suitable to direct gene expression in the selected host cell. The resulting chimeric genes can be introduced into the host microbial cell using techniques such as homologous recombination (Williams, J. G. K. (1988) *Methods Enzymol.* 167:766–778; Legarde, D. et al. (2000) *App. Environ. Microbiol.* 66:64–72). Host cells transformed with cDNAs for the instant HGGT polypeptides operably linked to functional promoters can then be analyzed for tocotrienol production using techniques described in Example 2.

EXAMPLE 10

Functional Conversion of a Homogentisate Phytyltransferase(HPT) into a Homogentisate Geranylgeranyl Transferase (HGGT) by Enzyme Engineering

Numerous examples exist of the conversion of the substrate specificity of one enzyme class to that of another by replacement of specific amino acid residues (e.g., Yuan, L. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:10639–10643; Cahoon, E. B. et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:4872–4877; Reznik, G. O. et al. (1998) *Proc. Nat. Acad. Sci. USA* 95:13525–13530). This conversion is typically achieved through comparisons of primary or tertiary structures of enzymes that have similar amino acid sequences but different substrate specificities. From these comparisons, one skilled in the art can recognize amino acid residues that likely contribute to the substrate recognition properties of a given functional class of enzymes. Residues that dictate substrate specificity are often ones that are conserved in members of a given functional class of enzyme but are different in other structurally related but functionally divergent classes (Yuan, L. et al., supra). By replacement of these residues, one can experimentally test whether a given amino acid or group of amino acids contribute to the substrate specificity of an enzyme.

Selected amino acid residues in an enzyme can be replaced through a variety of mutagenesis methods that are well known to those skilled in the art (e.g., Cahoon, E. B. et al., supra). By expressing the gene or cDNA for the mutant enzyme in a host cell, one can readily monitor whether replacement of amino acids has altered substrate specificity of the enzyme. Alteration of substrate specificity can be

measured by supplying the mutant enzyme with alternative substrates in an in vivo or in vitro assay. Alternatively, one skilled in the art can measure the accumulation of metabolic products of the mutated enzyme upon expression in a host cell.

As described in the instant invention, homogentisate geranylgeranyl transferases (HGGTs) (SEQ ID NOS: 2, 4, 6, 8 and 10) are members of the UbiA prenyltransferase family. Of the functionally diverse members of this family, HGGTs share the highest degree of amino acid sequence identity with homogentisate phytyltransferases (HPTs) (typically 40 to 50% identity). Despite this degree of structural relatedness, HGGTs and HPTs have divergent substrate specificities. HGGTs catalyze the condensation of homogentisate and geranylgeranyl pyrophosphate, the first committed step in tocotrienol biosynthesis. In contrast, HPTs catalyze the condensation of homogentisate and phytyl pyrophosphate, the first committed step in tocopherol biosynthesis. Sequence alignments between SEQ ID NOs: 2, 4, 6, 8, 10, 13, 14, 15, 16 and the sequence from *Synechocystis* sp. PCC 6803 (NCBI General Identification (GI) No. 16330366; SEQ ID NO: 17), the sequence from *Escherichia coli* K12 (4-hydroxybenzoate-octaprenyltransferase) (NCBI General Identification (GI) No. 16131866; SEQ ID NO: 18) and the sequence from oat (*Avena sativa*) (chlorophyll synthase) (NCBI General Identification (GI) No. 7378659; SEQ ID NO: 19) showed amino acid residues that are conserved in all HGGTs but are different in other UbiA prenyltranferase family members including HPTs. Using the sequence of the barley HGGT (SEQ ID NO: 2) as the basis for amino acid numbering, these residues include the following: arginine 72, glutamine 73, cysteine 85, cysteine 118, phenylalanine 124, isoleucine 127, isoleucine 128, glycine 129, threonine 131, proline 137, aspartate 142, phenylalanine 144, threonine 145, cysteine 161, isoleucine 213, methionine 270, glutamine 272, leucine 279, alanine 280, isoleucine 333, threonine 338, threonine 351, glutamine 355, serine 361, glycine 364, leucine 365, glutamate 380, asparagine 381 and phenylalanine 401. It is likely that these residues or some subgroup of these residues define the substrate specificity of HGGTs. As such, these amino acids represent targets for the functional conversion of an HPT-type enzyme into an HGGT. In achieving HGGT activity, one need not alter the HPT or UbiA prenyltransferase sequence to comprise all 29 of these residues at these positions. Rather, altering the target sequence to comprise at least any 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, or 29 of these residues at the positions identified can confer HGGT activity upon the target sequence.

By replacement of the equivalent residues (shown above) in an HPT into those found in HGGTs, an HPT can be converted into an enzyme that displays enhanced substrate specificity for geranylgeranyl pyrophosphate in preference to phytyl pyrophosphate. An HPT engineered in this manner is thus the functional equivalent of a naturally occurring HGGT and can be used to produce tocotrienols and increase the tocol content in transgenic host cells as described for HGGTs in Examples 2, 3, 4, 5, 6, 8 and 9. Thus, the invention described herein contemplates HGGTs obtained from plants, including monocots, and sequences from any source that have been engineered to function in plants, and more specifically, in monocots. The term plant homogentisate geranylgeranyl transferase encompasses homogentisate geranylgeranyl transferase sequences derived from a plant source in addition to HPT or other UbiA prenyltransferase sequences from any source modified to be or to encode a functional homogentisate geranylgeranyl transferase in a plant or plant cell using the methods taught herein. The term monocot homogentisate geranylgeranyl transferase encompasses homogentisate geranylgeranyl transferase seqeunces derived from a monocot plant source in addition to HPT or other UbiA prenyltransferase sequences from any source modified to be or to encode a functional homogentisate geranylgeranyl transferase in a monocot plant or plant cell using the methods taught herein

EXAMPLE 11

Tocotrienol Production in *Arabidopsis thaliana* by Transgenic Expression of the Barley Homogentisate Geranylgeranyl Transferase

The cDNA for barley homogentisate geranylgeranyl transferase (HGGT) (SEQ ID NO: 1) was constitutively expressed in *Arabidopsis thaliana* to further demonstrate the utility of this cDNA for tocotrienol production in transgenic plants. Plasmid pSH24 was used for *Agrobacterium*-mediated transformation of *Arabidopsis*. This plasmid, which was described in Example 2, contains the open reading frame of the barley HGGT operably linked on its 5' end to the cauliflower mosaic virus 35S promoter and on its 3' end to the transcription termination sequence of the nopaline synthase gene. This expression cassette together with a kanamycin resistance gene for transgenic plant selection is contained within right and left border regions of the plasmid. pSH24 was introduced into *Agrobacterium tumefaciens* strain C58C1-pMP90 using a freezethaw method of bacterial transformation (Holsters, M. et al. (1978) Mol. Gen. Genet 163(2):181–187). *Arabidopsis thaliana* cv Columbia plants were then transformed with *Agrobacterium* harboring pSH24 using the vacuum infiltration method described by Bechtold, N. et al. ((1993) *C. R. Acad. Sci., Paris* 316: 1194–1199). Transformed plants were selected for the ability of seeds from the infiltrated plants to germinate and for the seedlings to subsequently grow on media containing 40 μg/mL of kanamycin.

Leaves from the tplants transformed with the barley HGGT cDNA linked to the cauliflower mosaic virus 35S promoter were extracted and tocotrienol accumulation measured using analytical methods described in Example 2. Leaves were collected from the second whorl of 30-day old plants. Plants were maintained under a 14 h-22° C./10 h-18° C. light/dark cycle with a light intensity of 100 μmol m-2 s-1. In the organic extract from leaves of the transgenic plants, several tocotrienol molecular species were detected including α-, β-, and δ-tocotrienols. The primary tocol form found in these leaves was γ-tocotrienol. No tocotrienols, in contrast, were detected in untransformed plants, and the major tocol species in leaves of these plants instead was α-tocopherol. In addition, the tocol content of leaves of transformed plants was increased by as much as 10 to 5-fold relative to leaves of untransformed plants.

Leaves of T1 plants were found to accumulate large amounts of tocotrienols, which were absent from leaves of non-transformed plants. The content of Vitamin E antioxidants in leaves of segregating T2 plants from one of the selected lines was examined in detail. Leaves from plants displaying a null phenotype accumulated tocopherols (almost exclusively as a-tocopherol) to amounts of 40 to 60 mg/g dry weight. (Similar levels of tocopherols were detected in non-transformed plants.) In contrast, the total content of tocopherols and tocotrienols in leaves of phenotypepositive plants ranged from 700 to 900 mg/g dry weight. In leaves of these plants, gtocotrienol accounted for approximately 85% of the total Vitamin E content.

EXAMPLE 12

Identification of Protein Sequences Specific to HGGT Homologs

HGGT polypeptides described in the instant invention are members of the UbiA prenyltransferase family, which includes a number of functionally diverse enzymes such as chlorophyll synthase and 4-hydroxybenzoate octaprenyltransferase. Members of this family are distinguished by the presence of a UbiA consensus motif. Of the known members of this family, HGGTs are most closely related to HPTs. HGGTs described in the instant invention (SEQ ID NOS: 2, 4, 6, 8 and 10) share 40 to 50% identity with previously disclosed HPTs (SEQ ID NOs: 13–16). Using amino acid sequence alignments, one skilled in the art can readily distinguish HGGT polypeptides from HPT polypeptides by the presence of amino acid residues that are uniquely conserved in HGGTs. Such residues include (using SEQ ID NO: 2 as the basis for amino acid numbering): arginine 72, glutamine 73, cysteine 85, cysteine 118, phenylalanine 124, isoleucine 127, isoleucine 128, glycine 129, threonine 131, proline 137, aspartate 142, phenylalanine 144, threonine 145, cysteine 161, isoleucine 213, methionine 270, glutamine 272, leucine 279, alanine 280, isoleucine 333, threonine 338, threonine 351, glutamine 355, glycine 364, leucine 365, asparagine 381 and phenylalanine 401.

Protein motifs can be defined as short regions of conserved amino acid sequences that comprise part of a longer sequence. One skilled in the art can discern several HGGT-specific protein motifs. Using the barley HGGT amino acid sequence as the basis for numbering (SEQ ID 2), HGGT-specific motifs include "FXXIIGXT" which encompasses amino acids 124 through 131 and "(K/R)XXXDXFT" which encompasses amino acids 139 through 145. (Note: "X" indicates that a residue is not conserved in HGGTs or is not uniquely conserved in HGGTs.)

One skilled in the art can use amino acid sequence alignments such as those described above to identify "new" HGGTs that correspond to an isolated polypeptide or are deduced from an isolated nucleic acid fragment. An amino acid sequence of an HGGT polypeptide would be expected to be related to UbiA prenyltransferases and of highest relation to known HPT and HGGT polypeptides. In addition, an HGGT polypeptide would be expected to contain one or more of the protein motifs described above or one or more of the amino acid residues that are uniquely conserved in HGGT sequences disclosed in the instant invention as detailed above. Isolation of nucleic acid fragments encoding HGGTs can be achieved through a variety of techniques including hybridization with nucleic acid fragments encoding portions HGGT-related polypeptides or PCR-based strategies such as those described in the Examples herein. The biological source of the isolated nucleic acid fragments would preferably be a plant, plant tissue, or microbe that is known to produce tocotrienols. An isolated nucleic acid fragment can then be expressed in a tissue or cell, preferably a plant tissue or cell, that does not contain tocotrienol to determine whether it encodes a functional HGGT. Methods of expression in a host tissue or cell can include those described in Examples 2, 3, 4, 5, 6, 8, 9, and 11. Expression of a functional HGGT would be expected to confer the ability to synthesize tocotrienols to the tissue or cell. Accumulation of tocotrienols can be determined using analytical methods described in Example 2.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 68

<210> SEQ ID NO 1
<211> LENGTH: 1457
<212> TYPE: DNA
<213> ORGANISM: Hordeum vulgare
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (63)...(1286)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 1410, 1421
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 1

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gg atg caa gcc gtc acg gcg gcg gcc gcg gcg ggg cag ctg cta aca        107
   Met Gln Ala Val Thr Ala Ala Ala Ala Ala Gly Gln Leu Leu Thr
    1               5                  10                  15

gat acg agg aga ggg ccc aga tgt agg gct cgg ctg gga acg acg aga       155
Asp Thr Arg Arg Gly Pro Arg Cys Arg Ala Arg Leu Gly Thr Thr Arg
                20                  25                  30
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-continued

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tta tcc tgg aca ggt cga ttt gca gtg gaa gct ttt gca ggc cag tgc      203
Leu Ser Trp Thr Gly Arg Phe Ala Val Glu Ala Phe Ala Gly Gln Cys
             35                  40                  45

caa agt gct act act gta atg cat aaa ttc agt gcc att tct caa gct      251
Gln Ser Ala Thr Thr Val Met His Lys Phe Ser Ala Ile Ser Gln Ala
         50                  55                  60

gct agg cct aga aga aac aca aag aga cag tgc agc gat gat tat cca      299
Ala Arg Pro Arg Arg Asn Thr Lys Arg Gln Cys Ser Asp Asp Tyr Pro
     65                  70                  75

gcc ctc caa gct gga tgc agc gag gtt aat tgg gat caa aac ggt tcc      347
Ala Leu Gln Ala Gly Cys Ser Glu Val Asn Trp Asp Gln Asn Gly Ser
 80                  85                  90                  95

aac gcc aat cgg ctt gag gaa atc agg gga gat gtt ttg aag aaa ttg      395
Asn Ala Asn Arg Leu Glu Glu Ile Arg Gly Asp Val Leu Lys Lys Leu
                100                 105                 110

cgc tct ttc tat gaa ttt tgc agg cca cac aca att ttt ggc act ata      443
Arg Ser Phe Tyr Glu Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile
            115                 120                 125

ata ggt ata act tca gtg tct ctc ctg cca atg aag agc ata gat gat      491
Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Met Lys Ser Ile Asp Asp
        130                 135                 140

ttt act gtc acg gta cta cga gga tat ctc gag gct ttg act gct gct      539
Phe Thr Val Thr Val Leu Arg Gly Tyr Leu Glu Ala Leu Thr Ala Ala
    145                 150                 155

tta tgt atg aac att tat gtg gtc ggg ctg aat cag cta tat gac att      587
Leu Cys Met Asn Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile
160                 165                 170                 175

cag att gac aag atc aac aag cca ggt ctt cca ttg gca tct ggg gaa      635
Gln Ile Asp Lys Ile Asn Lys Pro Gly Leu Pro Leu Ala Ser Gly Glu
                180                 185                 190

ttt tca gta gca act gga gtt ttc tta gta ctc gca ttc ctg atc atg      683
Phe Ser Val Ala Thr Gly Val Phe Leu Val Leu Ala Phe Leu Ile Met
            195                 200                 205

agc ttt agc ata gga ata cgt tcc gga tcg gcg cca ctg atg tgt gct      731
Ser Phe Ser Ile Gly Ile Arg Ser Gly Ser Ala Pro Leu Met Cys Ala
        210                 215                 220

tta att gtc agc ttc ctt ctt gga agt gcg tac tcc att gag gct ccg      779
Leu Ile Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro
    225                 230                 235

ttc ctc cgg tgg aaa cgg cac gcg ctc ctc gct gca tca tgt atc cta      827
Phe Leu Arg Trp Lys Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu
240                 245                 250                 255

ttt gtg agg gct atc ttg gtc cag ttg gct ttc ttt gca cat atg cag      875
Phe Val Arg Ala Ile Leu Val Gln Leu Ala Phe Phe Ala His Met Gln
                260                 265                 270

caa cat gtt ctg aaa agg cca ttg gca gca acc aaa tcg ctg gtg ttt      923
Gln His Val Leu Lys Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe
            275                 280                 285

gca aca ttg ttt atg tgt tgc ttc tct gcc gtc ata gca cta ttc aag      971
Ala Thr Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys
        290                 295                 300

gat att cca gat gtt gat gga gat cga gac ttt ggt atc caa tcc ttg     1019
Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu
    305                 310                 315

agt gtg aga ttg ggg cct caa aga gtg tat cag ctc tgc ata agc ata     1067
Ser Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile
320                 325                 330                 335

ttg ttg aca gcc tat ggc gct gcc act cta gta gga gct tca tcc aca     1115
Leu Leu Thr Ala Tyr Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr
                340                 345                 350
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```
aac cta ttt caa aag atc atc act gtg tct ggt cat ggc ctg ctt gct     1163
Asn Leu Phe Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala
            355                 360                 365

ttg aca ctt tgg cag aga gcg cag cac ttt gag gtt gaa aac caa gcg     1211
Leu Thr Leu Trp Gln Arg Ala Gln His Phe Glu Val Glu Asn Gln Ala
        370                 375                 380

cgt gtc aca tca ttt tac atg ttc att tgg aag cta ttc tat gca gag     1259
Arg Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu
    385                 390                 395

tat ttc ctt ata cca ttt gtg cag tga aatttgtaca agggccagca          1306
Tyr Phe Leu Ile Pro Phe Val Gln  *
400                 405

gatgtgaact atatatacat gtaaaacaaa ttatattact gatgatactc aatccaatgc   1366

ttggattttg cttgtactgt gctatctgta atttcatgat ctanagaaag agcanatgtt   1426

ggatgtgtaa aaaaaaaaaa aaaaaaaaaa a                                  1457


<210> SEQ ID NO 2
<211> LENGTH: 407
<212> TYPE: PRT
<213> ORGANISM: Hordeum vulgare

<400> SEQUENCE: 2

Met Gln Ala Val Thr Ala Ala Ala Ala Ala Gly Gln Leu Leu Thr Asp
 1               5                  10                  15

Thr Arg Arg Gly Pro Arg Cys Arg Ala Arg Leu Gly Thr Thr Arg Leu
            20                  25                  30

Ser Trp Thr Gly Arg Phe Ala Val Glu Ala Phe Ala Gly Gln Cys Gln
        35                  40                  45

Ser Ala Thr Thr Val Met His Lys Phe Ser Ala Ile Ser Gln Ala Ala
    50                  55                  60

Arg Pro Arg Arg Asn Thr Lys Arg Gln Cys Ser Asp Asp Tyr Pro Ala
65                  70                  75                  80

Leu Gln Ala Gly Cys Ser Glu Val Asn Trp Asp Gln Asn Gly Ser Asn
                85                  90                  95

Ala Asn Arg Leu Glu Glu Ile Arg Gly Asp Val Leu Lys Lys Leu Arg
            100                 105                 110

Ser Phe Tyr Glu Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile
        115                 120                 125

Gly Ile Thr Ser Val Ser Leu Leu Pro Met Lys Ser Ile Asp Asp Phe
    130                 135                 140

Thr Val Thr Val Leu Arg Gly Tyr Leu Glu Ala Leu Thr Ala Ala Leu
145                 150                 155                 160

Cys Met Asn Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln
                165                 170                 175

Ile Asp Lys Ile Asn Lys Pro Gly Leu Pro Leu Ala Ser Gly Glu Phe
            180                 185                 190

Ser Val Ala Thr Gly Val Phe Leu Val Leu Ala Phe Leu Ile Met Ser
        195                 200                 205

Phe Ser Ile Gly Ile Arg Ser Gly Ser Ala Pro Leu Met Cys Ala Leu
    210                 215                 220

Ile Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro Phe
225                 230                 235                 240

Leu Arg Trp Lys Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu Phe
                245                 250                 255
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```
Val Arg Ala Ile Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln
            260                 265                 270

His Val Leu Lys Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe Ala
        275                 280                 285

Thr Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys Asp
    290                 295                 300

Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu Ser
305                 310                 315                 320

Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile Leu
                325                 330                 335

Leu Thr Ala Tyr Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr Asn
            340                 345                 350

Leu Phe Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala Leu
        355                 360                 365

Thr Leu Trp Gln Arg Ala Gln His Phe Glu Val Glu Asn Gln Ala Arg
    370                 375                 380

Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr
385                 390                 395                 400

Phe Leu Ile Pro Phe Val Gln
                405
```

<210> SEQ ID NO 3
<211> LENGTH: 1365
<212> TYPE: DNA
<213> ORGANISM: Triticum aestivum
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (53)...(1279)

<400> SEQUENCE: 3

```
ctttcacaca gatcccaggc cgcttttctc ctccggtggc cgcccggcga gg atg caa     58
                                                          Met Gln
                                                           1

gcc acc acg gcc gcg gcg gcg gcg cag ctg cta aca gat acg agg aga      106
Ala Thr Thr Ala Ala Ala Ala Ala Gln Leu Leu Thr Asp Thr Arg Arg
 5                  10                  15

ggg ccc aga tgt agt agg gct cgg ctg gga gcg acg aga tta tcc tgg      154
Gly Pro Arg Cys Ser Arg Ala Arg Leu Gly Ala Thr Arg Leu Ser Trp
 20                  25                  30

cca ggt cga ttt gca gtg gaa gct ttt gca ggc cgg tgc caa agc agt      202
Pro Gly Arg Phe Ala Val Glu Ala Phe Ala Gly Arg Cys Gln Ser Ser
 35                  40                  45                  50

gct act act gtc acg cat aga ttc agt gcc att tct caa gct aca agc      250
Ala Thr Thr Val Thr His Arg Phe Ser Ala Ile Ser Gln Ala Thr Ser
                55                  60                  65

cct aga aga aag gca agg agg cag tgc agc gat gat cag tca gcc ctc      298
Pro Arg Arg Lys Ala Arg Arg Gln Cys Ser Asp Asp Gln Ser Ala Leu
            70                  75                  80

caa gct gga tgc agc aag gtt aat cgc gat caa cat ggt tac gac gtg      346
Gln Ala Gly Cys Ser Lys Val Asn Arg Asp Gln His Gly Tyr Asp Val
 85                  90                  95

aac tgg ttt gag gaa atc agc caa gaa gtt tcg aag aaa ttg cgc gct      394
Asn Trp Phe Glu Glu Ile Ser Gln Glu Val Ser Lys Lys Leu Arg Ala
100                 105                 110

ttc tac cag ttc tgc aga cca cac aca atc ttt ggc act atc ata ggc      442
Phe Tyr Gln Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly
115                 120                 125                 130
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ata act tca gtg tct ctc ctg cca atg aag agc ata gat gat ttt act      490
Ile Thr Ser Val Ser Leu Leu Pro Met Lys Ser Ile Asp Asp Phe Thr
                135                 140                 145

gca acg gta cta aaa ggg tat ctc gag gct ttg gct gct gct tta tgt      538
Ala Thr Val Leu Lys Gly Tyr Leu Glu Ala Leu Ala Ala Ala Leu Cys
            150                 155                 160

atg aac att tat gtg gta ggg ctg aat cag cta tat gac att cag att      586
Met Asn Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile
        165                 170                 175

gac aag atc aac aag cca ggt ctt cca ttg gca gct ggg gaa ttt tca      634
Asp Lys Ile Asn Lys Pro Gly Leu Pro Leu Ala Ala Gly Glu Phe Ser
    180                 185                 190

gta gca act ggg gta ttt tta gta gtc aca ttc ctg atc atg agc ttt      682
Val Ala Thr Gly Val Phe Leu Val Val Thr Phe Leu Ile Met Ser Phe
195                 200                 205                 210

agc atc gga ata cat tcc gga tcg gtg cca ctg atg tat gct tta gtt      730
Ser Ile Gly Ile His Ser Gly Ser Val Pro Leu Met Tyr Ala Leu Val
                215                 220                 225

gtc agc ttc ctt ctt gga agt gca tac tcc att gag gct ccg ttg ctc      778
Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro Leu Leu
            230                 235                 240

cgg tgg aaa cgg cac gca ctc ctc gct gca tcc tgt atc cta ttt gtg      826
Arg Trp Lys Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu Phe Val
        245                 250                 255

agg gct atc ttg gtc cag ttg gct ttc ttt gca cat atg cag caa cat      874
Arg Ala Ile Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His
    260                 265                 270

gtt ctg aaa agg ccc ttg gca gca aca aaa tca ctg gtg ttt gca aca      922
Val Leu Lys Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe Ala Thr
275                 280                 285                 290

ttg ttc atg tgt tgc ttc tct gcc gtc ata gct cta ttc aag gat ata      970
Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys Asp Ile
                295                 300                 305

cct gat gtt gat gga gac cga gat ttt ggc atc caa tcc ttg agt gtg     1018
Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu Ser Val
            310                 315                 320

aga ttg ggg cca caa aga gtg tat cag ctc tgc ata agc ata ctg ttg     1066
Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile Leu Leu
        325                 330                 335

aca gcc tat ttg gct gcc act gta gta gga gct tca tcc aca cac cta     1114
Thr Ala Tyr Leu Ala Ala Thr Val Val Gly Ala Ser Ser Thr His Leu
    340                 345                 350

ctt caa aag ata atc act gtg tct ggt cat ggc ctg ctt gca cta aca     1162
Leu Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala Leu Thr
355                 360                 365                 370

ctt tgg cag aga gcg cgg cac ctt gag gtt gaa aat caa gcg cgt gtc     1210
Leu Trp Gln Arg Ala Arg His Leu Glu Val Glu Asn Gln Ala Arg Val
                375                 380                 385

aca tca ttt tac atg ttc att tgg aag cta ttc tat gca gag tat ttc     1258
Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe
            390                 395                 400

ctt ata cca ttt gtg cag tga aatttgtaca agggccagca gatgtgagct       1309
Leu Ile Pro Phe Val Gln  *
        405

atatatacat gtaaaacaaa ttatattact gatgataccc tatccaatgc ttggaa      1365
```

<210> SEQ ID NO 4
<211> LENGTH: 408
<212> TYPE: PRT
<213> ORGANISM: Triticum aestivum

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<400> SEQUENCE: 4

```
Met Gln Ala Thr Thr Ala Ala Ala Ala Ala Gln Leu Leu Thr Asp Thr
 1               5                  10                  15

Arg Arg Gly Pro Arg Cys Ser Arg Ala Arg Leu Gly Ala Thr Arg Leu
            20                  25                  30

Ser Trp Pro Gly Arg Phe Ala Val Glu Ala Phe Ala Gly Arg Cys Gln
        35                  40                  45

Ser Ser Ala Thr Thr Val Thr His Arg Phe Ser Ala Ile Ser Gln Ala
    50                  55                  60

Thr Ser Pro Arg Arg Lys Ala Arg Arg Gln Cys Ser Asp Asp Gln Ser
65                  70                  75                  80

Ala Leu Gln Ala Gly Cys Ser Lys Val Asn Arg Asp Gln His Gly Tyr
                85                  90                  95

Asp Val Asn Trp Phe Glu Glu Ile Ser Gln Glu Val Ser Lys Lys Leu
            100                 105                 110

Arg Ala Phe Tyr Gln Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile
        115                 120                 125

Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Met Lys Ser Ile Asp Asp
    130                 135                 140

Phe Thr Ala Thr Val Leu Lys Gly Tyr Leu Glu Ala Leu Ala Ala Ala
145                 150                 155                 160

Leu Cys Met Asn Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile
                165                 170                 175

Gln Ile Asp Lys Ile Asn Lys Pro Gly Leu Pro Leu Ala Ala Gly Glu
            180                 185                 190

Phe Ser Val Ala Thr Gly Val Phe Leu Val Val Thr Phe Leu Ile Met
        195                 200                 205

Ser Phe Ser Ile Gly Ile His Ser Gly Ser Val Pro Leu Met Tyr Ala
    210                 215                 220

Leu Val Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro
225                 230                 235                 240

Leu Leu Arg Trp Lys Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu
                245                 250                 255

Phe Val Arg Ala Ile Leu Val Gln Leu Ala Phe Phe Ala His Met Gln
            260                 265                 270

Gln His Val Leu Lys Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe
        275                 280                 285

Ala Thr Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys
    290                 295                 300

Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu
305                 310                 315                 320

Ser Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile
                325                 330                 335

Leu Leu Thr Ala Tyr Leu Ala Ala Thr Val Val Gly Ala Ser Ser Thr
            340                 345                 350

His Leu Leu Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala
        355                 360                 365

Leu Thr Leu Trp Gln Arg Ala Arg His Leu Glu Val Glu Asn Gln Ala
    370                 375                 380

Arg Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu
385                 390                 395                 400

Tyr Phe Leu Ile Pro Phe Val Gln
                405
```

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<210> SEQ ID NO 5
<211> LENGTH: 1242
<212> TYPE: DNA
<213> ORGANISM: Oryza sativa
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (6)...(1220)

<400> SEQUENCE: 5

```
agacg atg caa gcc tca tcg gcg gcg gcg gcg gcg gcg tgc tcg gct atc     50
      Met Gln Ala Ser Ser Ala Ala Ala Ala Ala Ala Cys Ser Ala Ile
       1               5                  10                  15

aag ccg gcg gcg cat cag cac acc gtg caa gtc cag gaa gat aag agg       98
Lys Pro Ala Ala His Gln His Thr Val Gln Val Gln Glu Asp Lys Arg
                   20                  25                  30

gga tcg gaa ttc agg gct cgg ttt gga acg agg aaa ctg tcc tgg gga      146
Gly Ser Glu Phe Arg Ala Arg Phe Gly Thr Arg Lys Leu Ser Trp Gly
               35                  40                  45

ggt aaa ttg tcg gtg gaa aat tct gct cta cac cag tgt caa agt ctc      194
Gly Lys Leu Ser Val Glu Asn Ser Ala Leu His Gln Cys Gln Ser Leu
        50                  55                  60

aca aga agc ata agg agg caa aaa aga caa cat tct cca gtc ctc caa      242
Thr Arg Ser Ile Arg Arg Gln Lys Arg Gln His Ser Pro Val Leu Gln
    65                  70                  75

gtg aga tgc tat gcc att gct ggt gat cag cac gaa tcc atc gcc act      290
Val Arg Cys Tyr Ala Ile Ala Gly Asp Gln His Glu Ser Ile Ala Thr
80                  85                  90                  95

gag ttt gaa gaa att tgc aaa gaa gtt ccc cag aaa ctg gga gct ttt      338
Glu Phe Glu Glu Ile Cys Lys Glu Val Pro Gln Lys Leu Gly Ala Phe
                100                 105                 110

tat cgg ttt tgc cga ccc cac aca att ttt ggc act ata ata gga atc      386
Tyr Arg Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly Ile
            115                 120                 125

act tca gtt tct ctc ctg cca atg agg agc cta gat gat ttt act atg      434
Thr Ser Val Ser Leu Leu Pro Met Arg Ser Leu Asp Asp Phe Thr Met
        130                 135                 140

aaa gca tta tgg gga ttt ctt gag gct tta tcc tct tct tta tgt atg      482
Lys Ala Leu Trp Gly Phe Leu Glu Ala Leu Ser Ser Ser Leu Cys Met
    145                 150                 155

aat atc tat gtt gta ggc ctg aat caa cta tat gac atc cag att gat      530
Asn Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile Asp
160                 165                 170                 175

aag gtc aat aag ccc agc ctt ccg ttg gcg tca gga gaa ttt tca gtg      578
Lys Val Asn Lys Pro Ser Leu Pro Leu Ala Ser Gly Glu Phe Ser Val
                180                 185                 190

gca act gga gca gtg tta gta ctc acg tcc ttg atc atg agc att gcc      626
Ala Thr Gly Ala Val Leu Val Leu Thr Ser Leu Ile Met Ser Ile Ala
            195                 200                 205

att gga atc aga tcc aaa tca gct cct ttg tta tgt gct ttg ttt atc      674
Ile Gly Ile Arg Ser Lys Ser Ala Pro Leu Leu Cys Ala Leu Phe Ile
        210                 215                 220

agt ttc ttt ctt gga agt gca tac tct gtt gat gct ccg tta ctc cgg      722
Ser Phe Phe Leu Gly Ser Ala Tyr Ser Val Asp Ala Pro Leu Leu Arg
    225                 230                 235

tgg aaa agg aac gcg ttt ctc gct gca tct tgt ata cta ttt gta aga      770
Trp Lys Arg Asn Ala Phe Leu Ala Ala Ser Cys Ile Leu Phe Val Arg
240                 245                 250                 255

gct gtc tta gtt cag cta gct ttc ttt gca cat atg cag caa cat gtt      818
Ala Val Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val
                260                 265                 270
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```
ctg aag agg ccc ttg gca cca aca aag tcg gtg gtt ttc gca aca tta      866
Leu Lys Arg Pro Leu Ala Pro Thr Lys Ser Val Val Phe Ala Thr Leu
            275                 280                 285

ttc atg tgt tgc ttt tct tca gtt ata gct tta ttc aag gat att cca      914
Phe Met Cys Cys Phe Ser Ser Val Ile Ala Leu Phe Lys Asp Ile Pro
        290                 295                 300

gat att gat ggt gac aga cat ttt ggc gtc gaa tcc ctg agc gta cgt      962
Asp Ile Asp Gly Asp Arg His Phe Gly Val Glu Ser Leu Ser Val Arg
    305                 310                 315

ttg ggt cca gaa aga gtg tat tgg ctc tgc ata aac ata cta tta aca     1010
Leu Gly Pro Glu Arg Val Tyr Trp Leu Cys Ile Asn Ile Leu Leu Thr
320                 325                 330                 335

gca tat ggg gct gcc att ttg gct gga gca tca tct aca aat cta tgt     1058
Ala Tyr Gly Ala Ala Ile Leu Ala Gly Ala Ser Ser Thr Asn Leu Cys
                340                 345                 350

caa atg att atc acc gtt ttc ggc cat ggc ctg ctt gcc ttt gca ctt     1106
Gln Met Ile Ile Thr Val Phe Gly His Gly Leu Leu Ala Phe Ala Leu
            355                 360                 365

tgg cag aga gca cag cac tgt gac gtt gaa aac aag gcg tgg atc aca     1154
Trp Gln Arg Ala Gln His Cys Asp Val Glu Asn Lys Ala Trp Ile Thr
        370                 375                 380

tca ttt tac atg ttc att tgg aag ttg ttc tac gct gag tat ttc ctt     1202
Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu
    385                 390                 395

ata cca ttt gtg cag tga gcactatata cacaagggca ag                   1242
Ile Pro Phe Val Gln  *
400
```

<210> SEQ ID NO 6
<211> LENGTH: 404
<212> TYPE: PRT
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 6

```
Met Gln Ala Ser Ser Ala Ala Ala Ala Ala Ala Cys Ser Ala Ile Lys
 1               5                  10                  15

Pro Ala Ala His Gln His Thr Val Gln Val Gln Glu Asp Lys Arg Gly
            20                  25                  30

Ser Glu Phe Arg Ala Arg Phe Gly Thr Arg Lys Leu Ser Trp Gly Gly
        35                  40                  45

Lys Leu Ser Val Glu Asn Ser Ala Leu His Gln Cys Gln Ser Leu Thr
    50                  55                  60

Arg Ser Ile Arg Arg Gln Lys Arg Gln His Ser Pro Val Leu Gln Val
65                  70                  75                  80

Arg Cys Tyr Ala Ile Ala Gly Asp Gln His Glu Ser Ile Ala Thr Glu
                85                  90                  95

Phe Glu Glu Ile Cys Lys Glu Val Pro Gln Lys Leu Gly Ala Phe Tyr
            100                 105                 110

Arg Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly Ile Thr
        115                 120                 125

Ser Val Ser Leu Leu Pro Met Arg Ser Leu Asp Asp Phe Thr Met Lys
    130                 135                 140

Ala Leu Trp Gly Phe Leu Glu Ala Leu Ser Ser Ser Leu Cys Met Asn
145                 150                 155                 160

Ile Tyr Val Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile Asp Lys
                165                 170                 175

Val Asn Lys Pro Ser Leu Pro Leu Ala Ser Gly Glu Phe Ser Val Ala
            180                 185                 190
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```
Thr Gly Ala Val Leu Val Leu Thr Ser Leu Ile Met Ser Ile Ala Ile
        195                 200                 205

Gly Ile Arg Ser Lys Ser Ala Pro Leu Leu Cys Ala Leu Phe Ile Ser
    210                 215                 220

Phe Phe Leu Gly Ser Ala Tyr Ser Val Asp Ala Pro Leu Leu Arg Trp
225                 230                 235                 240

Lys Arg Asn Ala Phe Leu Ala Ala Ser Cys Ile Leu Phe Val Arg Ala
                245                 250                 255

Val Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val Leu
            260                 265                 270

Lys Arg Pro Leu Ala Pro Thr Lys Ser Val Val Phe Ala Thr Leu Phe
        275                 280                 285

Met Cys Cys Phe Ser Ser Val Ile Ala Leu Phe Lys Asp Ile Pro Asp
    290                 295                 300

Ile Asp Gly Asp Arg His Phe Gly Val Glu Ser Leu Ser Val Arg Leu
305                 310                 315                 320

Gly Pro Glu Arg Val Tyr Trp Leu Cys Ile Asn Ile Leu Leu Thr Ala
                325                 330                 335

Tyr Gly Ala Ala Ile Leu Ala Gly Ala Ser Ser Thr Asn Leu Cys Gln
            340                 345                 350

Met Ile Ile Thr Val Phe Gly His Gly Leu Leu Ala Phe Ala Leu Trp
        355                 360                 365

Gln Arg Ala Gln His Cys Asp Val Glu Asn Lys Ala Trp Ile Thr Ser
    370                 375                 380

Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu Ile
385                 390                 395                 400

Pro Phe Val Gln


<210> SEQ ID NO 7
<211> LENGTH: 1730
<212> TYPE: DNA
<213> ORGANISM: Zea mays
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (211)...(1353)

<400> SEQUENCE: 7

ccttgagccg ttccgctgcc attcgaccac caccgccacg gcggcgccga tgccgactac      60

aaccactcgc agagactacc gcctccagcc ccgccgcttc tcatctccac gcagccgtcc     120

gatggccaag cggctcgccg gtgccgacaa agaggtgctc gtcgaggtgg tgaggttcac     180

gcataagagc ggactgaggg gctgtgacgg cgg ctg gaa gga ttt cct ggc cca      234
                                  Arg Leu Glu Gly Phe Pro Gly Pro
                                   1               5

gaa cga cag gaa gtt tgg cgc gtc ggt gag cga ccc gag gaa gcg ctc      282
Glu Arg Gln Glu Val Trp Arg Val Gly Glu Arg Pro Glu Glu Ala Leu
     10                  15                  20

cag gga cgt gct gtt cgc ctt cct gca gac ctt ccc caa gga ttt cca      330
Gln Gly Arg Ala Val Arg Leu Pro Ala Asp Leu Pro Gln Gly Phe Pro
 25                  30                  35                  40

gaa gaa aca ctt gat gcc act agt ccg acg aga gcc acc gga aga caa      378
Glu Glu Thr Leu Asp Ala Thr Ser Pro Thr Arg Ala Thr Gly Arg Gln
                 45                  50                  55

cgc agg cat tcc tca gtc ccc aaa gtg agc tgc tgg gca gct gct cat      426
Arg Arg His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His
             60                  65                  70
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```
cac caa cac aat tct aac ccc cag cag ttt cag gcg att ggc ata cga      474
His Gln His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg
         75                  80                  85

atc gca aag acg ctg cat gcc ttc tat cag ttc tgc cga cca cac aca      522
Ile Ala Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr
     90                  95                 100

ata ttt gga acc ata ata ggc att act tcg gtg tct atc ctg cca gtg      570
Ile Phe Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Ile Leu Pro Val
105                 110                 115                 120

aag agc ctg gac gat ttt acg ttg ata gct ata tgg gga ttt ctc gag      618
Lys Ser Leu Asp Asp Phe Thr Leu Ile Ala Ile Trp Gly Phe Leu Glu
                125                 130                 135

gct ttg gcc gcc gca tta tgt atg aac gtt tat gta gta ggg ctg aac      666
Ala Leu Ala Ala Ala Leu Cys Met Asn Val Tyr Val Val Gly Leu Asn
            140                 145                 150

aag gtc aat aag cca acc ctc cca tta tcg ttc gga gag ttt tca atg      714
Lys Val Asn Lys Pro Thr Leu Pro Leu Ser Phe Gly Glu Phe Ser Met
        155                 160                 165

cca act gca gta ttg tta gta gtg gca ttc ttg gtc atg agc att agc      762
Pro Thr Ala Val Leu Leu Val Val Ala Phe Leu Val Met Ser Ile Ser
    170                 175                 180

atc gga ata aga tca aag tct gct cca ttg atg tgt gct ttg ctt gtt      810
Ile Gly Ile Arg Ser Lys Ser Ala Pro Leu Met Cys Ala Leu Leu Val
185                 190                 195                 200

tgc ttc ctt ctt gga agc gca tac ccc att gac gtc cca tta ctc cgg      858
Cys Phe Leu Leu Gly Ser Ala Tyr Pro Ile Asp Val Pro Leu Leu Arg
                205                 210                 215

tgg aag cga cat gct ttt cta gct gca ttc tgc ata atc ttt gtg agg      906
Trp Lys Arg His Ala Phe Leu Ala Ala Phe Cys Ile Ile Phe Val Arg
            220                 225                 230

cct gta gtg gtc cag tta gct ttc ttt gca cac atg cag caa cat gtt      954
Pro Val Val Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val
        235                 240                 245

ctg aag agg ccc ttg gca cct aca agg tcg gtg gtc ttt gca aca tgt     1002
Leu Lys Arg Pro Leu Ala Pro Thr Arg Ser Val Val Phe Ala Thr Cys
    250                 255                 260

ttc atg tgt tgc ttc gct gca gta ata gcg cta ttc aag gat att cct     1050
Phe Met Cys Cys Phe Ala Ala Val Ile Ala Leu Phe Lys Asp Ile Pro
265                 270                 275                 280

gat gtc gat gga gat aga gat ttc ggc att cag tcc atg act gta cga     1098
Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Met Thr Val Arg
                285                 290                 295

tta ggc caa cag aga gtg cat agg ctc tgc att aat att ctc atg aca     1146
Leu Gly Gln Gln Arg Val His Arg Leu Cys Ile Asn Ile Leu Met Thr
            300                 305                 310

gca tac gca gcc gca att ttg gta ggc gcg tca tct acg aac ctg tat     1194
Ala Tyr Ala Ala Ala Ile Leu Val Gly Ala Ser Ser Thr Asn Leu Tyr
        315                 320                 325

cag aag att gtc att gtg tct ggt cat ggc ttg ctt gcc tcc aca ctc     1242
Gln Lys Ile Val Ile Val Ser Gly His Gly Leu Leu Ala Ser Thr Leu
    330                 335                 340

tgg caa aga gca caa caa ttt gac att gag aat aag gat tgt atc aca     1290
Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn Lys Asp Cys Ile Thr
345                 350                 355                 360

caa ttt tat atg ttc att tgg aag tta ttc tac gcc gag tat ttt ctt     1338
Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu
                365                 370                 375

ata cca ttt gtg tag taaagaatca tgcgaagaac aacacccctg ctatagacat     1393
Ile Pro Phe Val  *
            380
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gtgaaggttt attgctaatg ttactctacc ccctgctata gacatgtgaa ggtttattgc 1453

taatgttact ctaccgaatg gtctgaatgt ctatgcgtca tttgaatgta atatgactat 1513

ttgttgtatc agggtaacaa ctggagcaaa tgtaccatgt atattaagca ttaatttaac 1573

tgcatcattt gtaccatgta tattatgact atgtatgaga tattgtctct tattagtact 1633

ggatgtgatg tgtcttatta tgactatgga tgagactttt gtgatgtaat tgatgagact 1693

atggttttaa atattgttat gtgattgtgt gtgagat 1730

<210> SEQ ID NO 8
<211> LENGTH: 380
<212> TYPE: PRT
<213> ORGANISM: Zea mays

<400> SEQUENCE: 8

Arg Leu Glu Gly Phe Pro Gly Pro Glu Arg Gln Glu Val Trp Arg Val
1 5 10 15

Gly Glu Arg Pro Glu Glu Ala Leu Gln Gly Arg Ala Val Arg Leu Pro
20 25 30

Ala Asp Leu Pro Gln Gly Phe Pro Glu Glu Thr Leu Asp Ala Thr Ser
35 40 45

Pro Thr Arg Ala Thr Gly Arg Gln Arg Arg His Ser Ser Val Pro Lys
50 55 60

Val Ser Cys Trp Ala Ala Ala His His Gln His Asn Ser Asn Pro Gln
65 70 75 80

Gln Phe Gln Ala Ile Gly Ile Arg Ile Ala Lys Thr Leu His Ala Phe
85 90 95

Tyr Gln Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly Ile
100 105 110

Thr Ser Val Ser Ile Leu Pro Val Lys Ser Leu Asp Asp Phe Thr Leu
115 120 125

Ile Ala Ile Trp Gly Phe Leu Glu Ala Leu Ala Ala Ala Leu Cys Met
130 135 140

Asn Val Tyr Val Val Gly Leu Asn Lys Val Asn Lys Pro Thr Leu Pro
145 150 155 160

Leu Ser Phe Gly Glu Phe Ser Met Pro Thr Ala Val Leu Leu Val Val
165 170 175

Ala Phe Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Ser Ala
180 185 190

Pro Leu Met Cys Ala Leu Leu Val Cys Phe Leu Leu Gly Ser Ala Tyr
195 200 205

Pro Ile Asp Val Pro Leu Leu Arg Trp Lys Arg His Ala Phe Leu Ala
210 215 220

Ala Phe Cys Ile Ile Phe Val Arg Pro Val Val Val Gln Leu Ala Phe
225 230 235 240

Phe Ala His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro Thr
245 250 255

Arg Ser Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala Val
260 265 270

Ile Ala Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe
275 280 285

Gly Ile Gln Ser Met Thr Val Arg Leu Gly Gln Gln Arg Val His Arg
290 295 300

Leu Cys Ile Asn Ile Leu Met Thr Ala Tyr Ala Ala Ala Ile Leu Val
305 310 315 320

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Gly Ala Ser Ser Thr Asn Leu Tyr Gln Lys Ile Val Ile Val Ser Gly
                325                 330                 335

His Gly Leu Leu Ala Ser Thr Leu Trp Gln Arg Ala Gln Gln Phe Asp
            340                 345                 350

Ile Glu Asn Lys Asp Cys Ile Thr Gln Phe Tyr Met Phe Ile Trp Lys
        355                 360                 365

Leu Phe Tyr Ala Glu Tyr Phe Leu Ile Pro Phe Val
    370                 375                 380


<210> SEQ ID NO 9
<211> LENGTH: 1769
<212> TYPE: DNA
<213> ORGANISM: Zea mays
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (356)...(1441)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 1206, 1207
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 9

ccacgcgtcc gcgaccacca ccaccacggt gccgccgacg ccgaccacaa ccactcgtag     60

agactaccgt ctccgccccc gctgcttctc gtctccacgc agccgtccga tggccaagcg    120

gctcgccggc gccaacaaag aggtagggga cggcatgcca acaaagcagc ccacccggac    180

tcggcaggcc gcctcgtttc gctaacccat ttgatctgcg ccgggtgctc gtcgaggtgg    240

tgaggttcac gcagaagagc ggactgaggg gctgtgacgg tggctggaag gatttcctgg    300

cccggaacga caggaagttt ggagcgtcgg tgagcgacca gaggaagcgc tctag gga     358
                                                            Gly
                                                             1

cgt gtt gtt cgc ctt cct aca gac ctt ccc caa gga ttt cca gaa gaa      406
Arg Val Val Arg Leu Pro Thr Asp Leu Pro Gln Gly Phe Pro Glu Glu
  5                  10                  15

aca ctt gat gcc act agt ccg acg aga gcc act gga agg caa cac agg      454
Thr Leu Asp Ala Thr Ser Pro Thr Arg Ala Thr Gly Arg Gln His Arg
 20                  25                  30

cat tcc tca gtc ccc aaa gtg agc tgc tgg gca gct gct cat cac caa      502
His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His His Gln
 35                  40                  45

cac aat tct aac ccc cag cag ttt cag gcg att ggc ata cga atc gca      550
His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg Ile Ala
 50                  55                  60                  65

aag acg ctg cat gcc ttt tat cag ttc tgc cga cca cac aca ata ttt      598
Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr Ile Phe
                70                  75                  80

gga acc ata ata ggc att act tcg gtg tct ctc ctg cca gtg aag agc      646
Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Val Lys Ser
            85                  90                  95

ctg gac gat ttt acg ttg ata gct ata tgg gga ttt ctc gag gct ttg      694
Leu Asp Asp Phe Thr Leu Ile Ala Ile Trp Gly Phe Leu Glu Ala Leu
        100                 105                 110

gcc gcc gca tta tgt atg aac gtt tat gta gta ggg ctg aac cag cta      742
Ala Ala Ala Leu Cys Met Asn Val Tyr Val Val Gly Leu Asn Gln Leu
    115                 120                 125

ttt gac att gag att gac aag gtc aat aag cca acc ctc cca tta gcg      790
Phe Asp Ile Glu Ile Asp Lys Val Asn Lys Pro Thr Leu Pro Leu Ala
130                 135                 140                 145
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tcc gga gag ttt tca gtg cca act gca gta ttg tta gta gtg gca ttc      838
Ser Gly Glu Phe Ser Val Pro Thr Ala Val Leu Leu Val Val Ala Phe
                150                 155                 160

ttg gtc atg agc att agc atc gga ata aga tca aag tgt gcg cca ttg      886
Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Cys Ala Pro Leu
            165                 170                 175

atg tgt gct ttg ctt gtt agc ttc ctt ctt gga agc gca tac tcc att      934
Met Cys Ala Leu Leu Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser Ile
        180                 185                 190

gac gtt cca tta ctc cga tgg aag cga cat gct ttt cta gct gca ttc      982
Asp Val Pro Leu Leu Arg Trp Lys Arg His Ala Phe Leu Ala Ala Phe
    195                 200                 205

tgc ata atc ttt gtg agg gct gta gtg gtc cgg tta gct ttc ttt gca     1030
Cys Ile Ile Phe Val Arg Ala Val Val Val Arg Leu Ala Phe Phe Ala
210                 215                 220                 225

cac atg cag caa cat gtt ctg aag agg ccc ttg gca cct aca agg tcg     1078
His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro Thr Arg Ser
                230                 235                 240

gtg gtc ttt gca aca tgt ttc atg tgt tgc ttc gct gca gta ata gcg     1126
Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala Val Ile Ala
            245                 250                 255

cta ttc aag gat att cct gat gtc gat gga gat aga gat ttc ggc att     1174
Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile
        260                 265                 270

cag tcc atg act gta cga tta ggc caa cag ann gag ctc tgc att aat     1222
Gln Ser Met Thr Val Arg Leu Gly Gln Gln Xaa Glu Leu Cys Ile Asn
    275                 280                 285

att ctc atg aca gca tac gca gtc aca att ttg gta gga gcg ttg tct     1270
Ile Leu Met Thr Ala Tyr Ala Val Thr Ile Leu Val Gly Ala Leu Ser
290                 295                 300                 305

acg aac ctg tat cag aag att gtc att gtg tct ggt cat ggc ttg ctt     1318
Thr Asn Leu Tyr Gln Lys Ile Val Ile Val Ser Gly His Gly Leu Leu
                310                 315                 320

gcc tcc aca ctc tgg caa aga gca caa caa ttt gac att gag aat aag     1366
Ala Ser Thr Leu Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn Lys
            325                 330                 335

gat tgt atc aca caa ttt tat atg ttc att tgg aag tta ttc tat gcc     1414
Asp Cys Ile Thr Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala
        340                 345                 350

gag tat ttt ctt ata cca ttt gtg tag taaagaatca tgcgaagaac           1461
Glu Tyr Phe Leu Ile Pro Phe Val  *
    355                 360

atcacccttg ctatagacat gtgaaggttc attgctaatg ttactctacc gaatggtctg   1521

aatgtctatg cgtcatttgt atgtaatatg actttgttgt atcagggtaa caactggagc   1581

aaatgtacca tgtatattaa gcattaattt agctgtgtca tttgtaccat gtatattatg   1641

actatgtatg agatattgtc tcttattagt actagatgtg atgtgtctta ttatgactat   1701

ggatgaaact tttgtgatgt aattgatgag actatggatt taaatattgt taaaaaaaaa   1761

aaaaaaaa                                                           1769
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<210> SEQ ID NO 10
<211> LENGTH: 361
<212> TYPE: PRT
<213> ORGANISM: Zea mays
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: 284
<223> OTHER INFORMATION: Xaa = Any Amino Acid

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<400> SEQUENCE: 10

Gly Arg Val Val Arg Leu Pro Thr Asp Leu Pro Gln Gly Phe Pro Glu
1 5 10 15

Glu Thr Leu Asp Ala Thr Ser Pro Thr Arg Ala Thr Gly Arg Gln His
20 25 30

Arg His Ser Ser Val Pro Lys Val Ser Cys Trp Ala Ala Ala His His
35 40 45

Gln His Asn Ser Asn Pro Gln Gln Phe Gln Ala Ile Gly Ile Arg Ile
50 55 60

Ala Lys Thr Leu His Ala Phe Tyr Gln Phe Cys Arg Pro His Thr Ile
65 70 75 80

Phe Gly Thr Ile Ile Gly Ile Thr Ser Val Ser Leu Leu Pro Val Lys
85 90 95

Ser Leu Asp Asp Phe Thr Leu Ile Ala Ile Trp Gly Phe Leu Glu Ala
100 105 110

Leu Ala Ala Ala Leu Cys Met Asn Val Tyr Val Val Gly Leu Asn Gln
115 120 125

Leu Phe Asp Ile Glu Ile Asp Lys Val Asn Lys Pro Thr Leu Pro Leu
130 135 140

Ala Ser Gly Glu Phe Ser Val Pro Thr Ala Val Leu Leu Val Val Ala
145 150 155 160

Phe Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Cys Ala Pro
165 170 175

Leu Met Cys Ala Leu Leu Val Ser Phe Leu Leu Gly Ser Ala Tyr Ser
180 185 190

Ile Asp Val Pro Leu Leu Arg Trp Lys Arg His Ala Phe Leu Ala Ala
195 200 205

Phe Cys Ile Ile Phe Val Arg Ala Val Val Val Arg Leu Ala Phe Phe
210 215 220

Ala His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro Thr Arg
225 230 235 240

Ser Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala Val Ile
245 250 255

Ala Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly
260 265 270

Ile Gln Ser Met Thr Val Arg Leu Gly Gln Gln Xaa Glu Leu Cys Ile
275 280 285

Asn Ile Leu Met Thr Ala Tyr Ala Val Thr Ile Leu Val Gly Ala Leu
290 295 300

Ser Thr Asn Leu Tyr Gln Lys Ile Val Ile Val Ser Gly His Gly Leu
305 310 315 320

Leu Ala Ser Thr Leu Trp Gln Arg Ala Gln Gln Phe Asp Ile Glu Asn
325 330 335

Lys Asp Cys Ile Thr Gln Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr
340 345 350

Ala Glu Tyr Phe Leu Ile Pro Phe Val
355 360

<210> SEQ ID NO 11
<211> LENGTH: 702
<212> TYPE: DNA
<213> ORGANISM: Hordeum vulgare
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)...(702)

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<400> SEQUENCE: 11

```
tac ctt gtt ggg ttg aat cag cta tat gac att cag att gac aag atc       48
Tyr Leu Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile Asp Lys Ile
 1               5                  10                  15

aac aag cca ggt ctt cca ttg gca tct ggg gaa ttt tca gta gca act       96
Asn Lys Pro Gly Leu Pro Leu Ala Ser Gly Glu Phe Ser Val Ala Thr
            20                  25                  30

gga gtt ttc tta gta ctc gca ttc ctg atc atg agc ttt agc ata gga      144
Gly Val Phe Leu Val Leu Ala Phe Leu Ile Met Ser Phe Ser Ile Gly
        35                  40                  45

ata cgt tcc gga tcg gcg cca ctg atg tgt gct tta att gtc agc ttc      192
Ile Arg Ser Gly Ser Ala Pro Leu Met Cys Ala Leu Ile Val Ser Phe
    50                  55                  60

ctt ctt gga agt gcg tac tcc att gag gct ccg ttc ctc cgg tgg aaa      240
Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro Phe Leu Arg Trp Lys
65                  70                  75                  80

cgg cac gcg ctc ctc gct gca tca tgt atc cta ttt gtg agg gct atc      288
Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu Phe Val Arg Ala Ile
                85                  90                  95

ttg gtc cag ttg gct ttc ttt gca cat atg cag caa cat gtt ctg aaa      336
Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val Leu Lys
            100                 105                 110

agg cca ttg gca gca acc aaa tcg ctg gtg ttt gca aca ttg ttt atg      384
Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe Ala Thr Leu Phe Met
        115                 120                 125

tgt tgc ttc tct gcc gtc ata gca cta ttc aag gat att cca gat gtt      432
Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Val
    130                 135                 140

gat gga gat cga gac ttt ggt atc caa tcc ttg agt gtg aga ttg ggg      480
Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu Ser Val Arg Leu Gly
145                 150                 155                 160

cct caa aga gtg tat cag ctc tgc ata agc ata ttg ttg aca gcc tat      528
Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile Leu Leu Thr Ala Tyr
                165                 170                 175

ggc gct gcc act cta gta gga gct tca tcc aca aac cta ttt caa aag      576
Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr Asn Leu Phe Gln Lys
            180                 185                 190

atc atc act gtg tct ggt cat ggc ctg ctt gct ttg aca ctt tgg cag      624
Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala Leu Thr Leu Trp Gln
        195                 200                 205

aga gcg cag cac ttt gag gtt gaa aac caa gcg cgt gtc aca tca ttt      672
Arg Ala Gln His Phe Glu Val Glu Asn Gln Ala Arg Val Thr Ser Phe
    210                 215                 220

tac atg ttc atc tgg aac ttg ttt tac gcg                             702
Tyr Met Phe Ile Trp Asn Leu Phe Tyr Ala
225                 230
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<210> SEQ ID NO 12
<211> LENGTH: 234
<212> TYPE: PRT
<213> ORGANISM: Hordeum vulgare

<400> SEQUENCE: 12

```
Tyr Leu Val Gly Leu Asn Gln Leu Tyr Asp Ile Gln Ile Asp Lys Ile
 1               5                  10                  15

Asn Lys Pro Gly Leu Pro Leu Ala Ser Gly Glu Phe Ser Val Ala Thr
            20                  25                  30

Gly Val Phe Leu Val Leu Ala Phe Leu Ile Met Ser Phe Ser Ile Gly
        35                  40                  45
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Ile Arg Ser Gly Ser Ala Pro Leu Met Cys Ala Leu Ile Val Ser Phe
    50                  55                  60

Leu Leu Gly Ser Ala Tyr Ser Ile Glu Ala Pro Phe Leu Arg Trp Lys
65                  70                  75                  80

Arg His Ala Leu Leu Ala Ala Ser Cys Ile Leu Phe Val Arg Ala Ile
                85                  90                  95

Leu Val Gln Leu Ala Phe Phe Ala His Met Gln Gln His Val Leu Lys
            100                 105                 110

Arg Pro Leu Ala Ala Thr Lys Ser Leu Val Phe Ala Thr Leu Phe Met
        115                 120                 125

Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Val
    130                 135                 140

Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu Ser Val Arg Leu Gly
145                 150                 155                 160

Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile Leu Leu Thr Ala Tyr
                165                 170                 175

Gly Ala Ala Thr Leu Val Gly Ala Ser Ser Thr Asn Leu Phe Gln Lys
            180                 185                 190

Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala Leu Thr Leu Trp Gln
        195                 200                 205

Arg Ala Gln His Phe Glu Val Glu Asn Gln Ala Arg Val Thr Ser Phe
    210                 215                 220

Tyr Met Phe Ile Trp Asn Leu Phe Tyr Ala
225                 230


<210> SEQ ID NO 13
<211> LENGTH: 393
<212> TYPE: PRT
<213> ORGANISM: Arabidopsis thaliana

<400> SEQUENCE: 13

Met Glu Ser Leu Leu Ser Ser Ser Ser Leu Val Ser Ala Ala Gly Gly
 1               5                  10                  15

Phe Cys Trp Lys Lys Gln Asn Leu Lys Leu His Ser Leu Ser Glu Ile
            20                  25                  30

Arg Val Leu Arg Cys Asp Ser Ser Lys Val Val Ala Lys Pro Lys Phe
        35                  40                  45

Arg Asn Asn Leu Val Arg Pro Asp Gly Gln Gly Ser Ser Leu Leu Leu
    50                  55                  60

Tyr Pro Lys His Lys Ser Arg Phe Arg Val Asn Ala Thr Ala Gly Gln
65                  70                  75                  80

Pro Glu Ala Phe Asp Ser Asn Ser Lys Gln Lys Ser Phe Arg Asp Ser
                85                  90                  95

Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr
            100                 105                 110

Val Leu Ser Ile Leu Ser Val Ser Phe Leu Ala Val Glu Lys Val Ser
        115                 120                 125

Asp Ile Ser Pro Leu Leu Phe Thr Gly Ile Leu Glu Ala Val Val Ala
    130                 135                 140

Ala Leu Met Met Asn Ile Tyr Ile Val Gly Leu Asn Gln Leu Ser Asp
145                 150                 155                 160

Val Glu Ile Asp Lys Val Asn Lys Pro Tyr Leu Pro Leu Ala Ser Gly
                165                 170                 175

Glu Tyr Ser Val Asn Thr Gly Ile Ala Ile Val Ala Ser Phe Ser Ile
            180                 185                 190
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Met Ser Phe Trp Leu Gly Trp Ile Val Gly Ser Trp Pro Leu Phe Trp
        195                 200                 205

Ala Leu Phe Val Ser Phe Met Leu Gly Thr Ala Tyr Ser Ile Asn Leu
    210                 215                 220

Pro Leu Leu Arg Trp Lys Arg Phe Ala Leu Val Ala Ala Met Cys Ile
225                 230                 235                 240

Leu Ala Val Arg Ala Ile Ile Val Gln Ile Ala Phe Tyr Leu His Ile
                245                 250                 255

Gln Thr His Val Phe Gly Arg Pro Ile Leu Phe Thr Arg Pro Leu Ile
            260                 265                 270

Phe Ala Thr Ala Phe Met Ser Phe Phe Ser Val Val Ile Ala Leu Phe
        275                 280                 285

Lys Asp Ile Pro Asp Ile Glu Gly Asp Lys Ile Phe Gly Ile Arg Ser
    290                 295                 300

Phe Ser Val Thr Leu Gly Gln Lys Arg Val Phe Trp Thr Cys Val Thr
305                 310                 315                 320

Leu Leu Gln Met Ala Tyr Ala Val Ala Ile Leu Val Gly Ala Thr Ser
                325                 330                 335

Pro Phe Ile Trp Ser Lys Val Ile Ser Val Val Gly His Val Ile Leu
            340                 345                 350

Ala Thr Thr Leu Trp Ala Arg Ala Lys Ser Val Asp Leu Ser Ser Lys
        355                 360                 365

Thr Glu Ile Thr Ser Cys Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala
    370                 375                 380

Glu Tyr Leu Leu Leu Pro Phe Leu Lys
385                 390


<210> SEQ ID NO 14
<211> LENGTH: 395
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 14

Met Asp Ser Met Leu Leu Arg Ser Phe Pro Asn Ile Asn Asn Ala Ser
 1               5                  10                  15

Ser Leu Ala Thr Thr Gly Ser Tyr Leu Pro Asn Ala Ser Trp His Asn
            20                  25                  30

Arg Lys Ile Gln Lys Glu Tyr Asn Phe Leu Arg Phe Arg Trp Pro Ser
        35                  40                  45

Leu Asn His His Tyr Lys Ser Ile Glu Gly Gly Cys Thr Cys Lys Lys
    50                  55                  60

Cys Asn Ile Lys Phe Val Val Lys Ala Thr Ser Glu Lys Ser Phe Glu
65                  70                  75                  80

Ser Glu Pro Gln Ala Phe Asp Pro Lys Ser Ile Leu Asp Ser Val Lys
                85                  90                  95

Asn Ser Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro His Thr Val Ile
            100                 105                 110

Gly Thr Ala Leu Ser Ile Ile Ser Val Ser Leu Leu Ala Val Glu Lys
        115                 120                 125

Ile Ser Asp Ile Ser Pro Leu Phe Phe Thr Gly Val Leu Glu Ala Val
    130                 135                 140

Val Ala Ala Leu Phe Met Asn Ile Tyr Ile Val Gly Leu Asn Gln Leu
145                 150                 155                 160

Ser Asp Val Glu Ile Asp Lys Ile Asn Lys Pro Tyr Leu Pro Leu Ala
                165                 170                 175
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Ser Gly Glu Tyr Ser Phe Glu Thr Gly Val Thr Ile Val Ala Ser Phe
            180                 185                 190

Ser Ile Leu Ser Phe Trp Leu Gly Trp Val Val Gly Ser Trp Pro Leu
        195                 200                 205

Phe Trp Ala Leu Phe Val Ser Phe Val Leu Gly Thr Ala Tyr Ser Ile
    210                 215                 220

Asn Val Pro Leu Leu Arg Trp Lys Arg Phe Ala Val Leu Ala Ala Met
225                 230                 235                 240

Cys Ile Leu Ala Val Arg Ala Val Ile Val Gln Leu Ala Phe Phe Leu
                245                 250                 255

His Ile Gln Thr His Val Tyr Lys Arg Pro Pro Val Phe Ser Arg Ser
            260                 265                 270

Leu Ile Phe Ala Thr Ala Phe Met Ser Phe Phe Ser Val Val Ile Ala
        275                 280                 285

Leu Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Lys Val Phe Gly Ile
    290                 295                 300

Gln Ser Phe Ser Val Arg Leu Gly Gln Lys Pro Val Phe Trp Thr Cys
305                 310                 315                 320

Val Ile Leu Leu Glu Ile Ala Tyr Gly Val Ala Leu Leu Val Gly Ala
                325                 330                 335

Ala Ser Pro Cys Leu Trp Ser Lys Ile Val Thr Gly Leu Gly His Ala
            340                 345                 350

Val Leu Ala Ser Ile Leu Trp Phe His Ala Lys Ser Val Asp Leu Lys
        355                 360                 365

Ser Lys Ala Ser Ile Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe
    370                 375                 380

Tyr Ala Glu Tyr Leu Leu Ile Pro Phe Val Arg
385                 390                 395


<210> SEQ ID NO 15
<211> LENGTH: 404
<212> TYPE: PRT
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 15

Met Asp Ser Leu Arg Leu Arg Pro Ser Leu Leu Ala Ala Arg Ala Pro
 1               5                  10                  15

Gly Ala Ala Ser Leu Pro Pro Leu Arg Arg Asp His Phe Leu Pro Pro
            20                  25                  30

Leu Cys Ser Ile His Arg Asn Gly Lys Arg Pro Val Ser Leu Ser Ser
        35                  40                  45

Gln Arg Thr Gln Gly Pro Ser Phe Asp Gln Cys Gln Lys Phe Phe Gly
    50                  55                  60

Trp Lys Ser Ser His His Arg Ile Pro His Arg Pro Thr Ser Ser Ser
65                  70                  75                  80

Ala Asp Ala Ser Gly Gln Pro Leu Gln Ser Ser Ala Glu Ala His Asp
                85                  90                  95

Ser Ser Ser Ile Trp Lys Pro Ile Ser Ser Ser Pro Asp Ala Phe Tyr
            100                 105                 110

Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr Ala Leu Ser Ile Val
        115                 120                 125

Ser Val Ser Leu Leu Ala Val Glu Asn Leu Ser Asp Val Ser Pro Leu
    130                 135                 140

Phe Leu Thr Gly Leu Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn
145                 150                 155                 160
```

-continued

```
Ile Tyr Ile Val Gly Leu Asn Gln Leu Phe Asp Ile Glu Ile Asp Lys
                165                 170                 175

Val Asn Lys Pro Thr Leu Pro Leu Ala Ser Gly Glu Tyr Ser Pro Ala
            180                 185                 190

Thr Gly Val Ala Leu Val Ser Ala Phe Ala Ala Met Ser Phe Gly Leu
        195                 200                 205

Gly Trp Ala Val Gly Ser Gln Pro Leu Phe Leu Ala Leu Phe Ile Ser
    210                 215                 220

Phe Ile Leu Gly Thr Ala Tyr Ser Ile Asn Leu Pro Phe Leu Arg Trp
225                 230                 235                 240

Lys Arg Ser Ala Val Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala
                245                 250                 255

Val Ile Val Gln Leu Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe
            260                 265                 270

Arg Arg Pro Ala Val Phe Thr Arg Pro Leu Ile Phe Ala Thr Ala Phe
        275                 280                 285

Met Thr Phe Phe Ser Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp
    290                 295                 300

Ile Glu Gly Asp Arg Ile Phe Gly Ile Lys Ser Phe Ser Val Arg Leu
305                 310                 315                 320

Gly Gln Lys Lys Val Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala
                325                 330                 335

Tyr Cys Val Ala Ile Leu Met Gly Ala Thr Ser Ala Cys Leu Trp Ser
            340                 345                 350

Lys Tyr Ala Thr Val Val Gly His Ala Ile Leu Ala Ala Ile Leu Trp
        355                 360                 365

Asn Arg Ser Arg Ser Ile Asp Leu Thr Ser Lys Thr Ala Ile Thr Ser
    370                 375                 380

Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile
385                 390                 395                 400

Pro Leu Val Arg
```

<210> SEQ ID NO 16
<211> LENGTH: 399
<212> TYPE: PRT
<213> ORGANISM: Zea mays

<400> SEQUENCE: 16

```
Met Asp Ala Leu Arg Leu Arg Pro Ser Leu Leu Pro Val Arg Pro Gly
 1               5                  10                  15

Ala Ala Arg Pro Arg Asp His Phe Leu Pro Pro Cys Cys Ser Ile Gln
            20                  25                  30

Arg Asn Gly Glu Gly Arg Ile Cys Phe Ser Ser Gln Arg Thr Gln Gly
        35                  40                  45

Pro Thr Leu His His His Gln Lys Phe Phe Glu Trp Lys Ser Ser Tyr
    50                  55                  60

Cys Arg Ile Ser His Arg Ser Leu Asn Thr Ser Val Asn Ala Ser Gly
65                  70                  75                  80

Gln Gln Leu Gln Ser Glu Pro Glu Thr His Asp Ser Thr Thr Ile Trp
                85                  90                  95

Arg Ala Ile Ser Ser Ser Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro
            100                 105                 110

His Thr Val Ile Gly Thr Ala Leu Ser Ile Val Ser Val Ser Leu Leu
        115                 120                 125
```

-continued

```
Ala Val Gln Ser Leu Ser Asp Ile Ser Pro Leu Phe Leu Thr Gly Leu
    130                 135                 140

Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn Ile Tyr Ile Val Gly
145                 150                 155                 160

Leu Asn Gln Leu Phe Asp Ile Glu Ile Asp Lys Val Asn Lys Pro Thr
                165                 170                 175

Leu Pro Leu Ala Ser Gly Glu Tyr Thr Leu Ala Thr Gly Val Ala Ile
            180                 185                 190

Val Ser Val Phe Ala Ala Met Ser Phe Gly Leu Gly Trp Ala Val Gly
        195                 200                 205

Ser Gln Pro Leu Phe Trp Ala Leu Phe Ile Ser Phe Val Leu Gly Thr
    210                 215                 220

Ala Tyr Ser Ile Asn Leu Pro Tyr Leu Arg Trp Lys Arg Phe Ala Val
225                 230                 235                 240

Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala Val Ile Val Gln Leu
                245                 250                 255

Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe Arg Arg Pro Ala Val
            260                 265                 270

Phe Ser Arg Pro Leu Leu Phe Ala Thr Gly Phe Met Thr Phe Phe Ser
        275                 280                 285

Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Arg
    290                 295                 300

Ile Phe Gly Ile Arg Ser Phe Ser Val Arg Leu Gly Gln Lys Lys Val
305                 310                 315                 320

Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala Tyr Ser Val Ala Ile
                325                 330                 335

Leu Met Gly Ala Thr Ser Ser Cys Leu Trp Ser Lys Thr Ala Thr Ile
            340                 345                 350

Ala Gly His Ser Ile Leu Ala Ala Ile Leu Trp Ser Cys Ala Arg Ser
        355                 360                 365

Val Asp Leu Thr Ser Lys Ala Ala Ile Thr Ser Phe Tyr Met Phe Ile
    370                 375                 380

Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile Pro Leu Val Arg
385                 390                 395


<210> SEQ ID NO 17
<211> LENGTH: 308
<212> TYPE: PRT
<213> ORGANISM: Synechocystis PCC6803

<400> SEQUENCE: 17

Met Ala Thr Ile Gln Ala Phe Trp Arg Phe Ser Arg Pro His Thr Ile
 1               5                  10                  15

Ile Gly Thr Thr Leu Ser Val Trp Ala Val Tyr Leu Leu Thr Ile Leu
            20                  25                  30

Gly Asp Gly Asn Ser Val Asn Ser Pro Ala Ser Leu Asp Leu Val Phe
        35                  40                  45

Gly Ala Trp Leu Ala Cys Leu Leu Gly Asn Val Tyr Ile Val Gly Leu
    50                  55                  60

Asn Gln Leu Trp Asp Val Asp Ile Asp Arg Ile Asn Lys Pro Asn Leu
65                  70                  75                  80

Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val
                85                  90                  95

Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu
            100                 105                 110
```

-continued

```
Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr
        115                 120                 125

Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu
    130                 135                 140

Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu
145                 150                 155                 160

Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile
                165                 170                 175

Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile
            180                 185                 190

Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln
        195                 200                 205

Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu
    210                 215                 220

Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp
225                 230                 235                 240

Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys
                245                 250                 255

Leu Leu Ala Leu Leu Trp Trp Arg Ser Arg Asp Val His Leu Glu Ser
            260                 265                 270

Lys Thr Glu Ile Ala Ser Phe Tyr Gln Phe Ile Trp Lys Leu Phe Phe
        275                 280                 285

Leu Glu Tyr Leu Leu Tyr Pro Leu Ala Leu Trp Leu Pro Asn Phe Ser
    290                 295                 300

Asn Thr Ile Phe
305
```

<210> SEQ ID NO 18
<211> LENGTH: 290
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli K12

<400> SEQUENCE: 18

```
Met Glu Trp Ser Leu Thr Gln Asn Lys Leu Leu Ala Phe His Arg Leu
1               5                  10                  15

Met Arg Thr Asp Lys Pro Ile Gly Ala Leu Leu Leu Leu Trp Pro Thr
            20                  25                  30

Leu Trp Ala Leu Trp Val Ala Thr Pro Gly Val Pro Gln Leu Trp Ile
        35                  40                  45

Leu Ala Val Phe Val Ala Gly Val Trp Leu Met Arg Ala Ala Gly Cys
    50                  55                  60

Val Val Asn Asp Tyr Ala Asp Arg Lys Phe Asp Gly His Val Lys Arg
65                  70                  75                  80

Thr Ala Asn Arg Pro Leu Pro Ser Gly Ala Val Thr Glu Lys Glu Ala
                85                  90                  95

Arg Ala Leu Phe Val Val Leu Val Leu Ile Ser Phe Leu Leu Val Leu
            100                 105                 110

Thr Leu Asn Thr Met Thr Ile Leu Leu Ser Ile Ala Ala Leu Ala Leu
        115                 120                 125

Ala Trp Val Tyr Pro Phe Met Lys Arg Tyr Thr His Leu Pro Gln Val
    130                 135                 140

Val Leu Gly Ala Ala Phe Gly Trp Ser Ile Pro Met Ala Phe Ala Ala
145                 150                 155                 160

Val Ser Glu Ser Val Pro Leu Ser Cys Trp Leu Met Phe Leu Ala Asn
                165                 170                 175
```

-continued

```
Ile Leu Trp Ala Val Ala Tyr Asp Thr Gln Tyr Ala Met Val Asp Arg
            180                 185                 190

Asp Asp Asp Val Lys Ile Gly Ile Lys Ser Thr Ala Ile Leu Phe Gly
        195                 200                 205

Gln Tyr Asp Lys Leu Ile Ile Gly Ile Leu Gln Ile Gly Val Leu Ala
    210                 215                 220

Leu Met Ala Ile Ile Gly Glu Leu Asn Gly Leu Gly Trp Gly Tyr Tyr
225                 230                 235                 240

Trp Ser Ile Leu Val Ala Gly Ala Leu Phe Val Tyr Gln Gln Lys Leu
                245                 250                 255

Ile Ala Asn Arg Glu Arg Glu Ala Cys Phe Lys Ala Phe Met Asn Asn
            260                 265                 270

Asn Tyr Val Gly Leu Val Leu Phe Leu Gly Leu Ala Met Ser Tyr Trp
        275                 280                 285

His Phe
    290


<210> SEQ ID NO 19
<211> LENGTH: 378
<212> TYPE: PRT
<213> ORGANISM: Avena sativa

<400> SEQUENCE: 19

Met Ala Thr Ser His Pro Leu Ala Ala Ala Ala Thr Ser Ser Ser
 1               5                  10                  15

Ser Ala Thr Phe Arg Pro Pro Leu Arg Phe Leu Ser Ser Pro Pro Ser
            20                  25                  30

Ser Leu Thr Leu Asn Arg Arg Arg Ser Phe Pro Val Val Cys Ala Ala
        35                  40                  45

Asp Ala Asp Ala Lys Glu Thr Thr Lys Lys Pro Thr Ile Pro Asp Lys
    50                  55                  60

Ala Pro Ala Ala Gly Ser Ser Phe Asn Gln Leu Leu Gly Ile Lys Gly
65                  70                  75                  80

Ala Lys Gln Glu Thr Asn Ile Trp Lys Ile Arg Leu Gln Leu Thr Lys
                85                  90                  95

Pro Val Thr Trp Pro Pro Leu Val Trp Gly Val Leu Cys Gly Ala Ala
            100                 105                 110

Ala Ser Gly Asn Phe His Trp Thr Val Glu Asp Val Thr Lys Ser Ile
        115                 120                 125

Val Cys Met Leu Met Ser Gly Pro Cys Leu Thr Gly Tyr Thr Gln Thr
    130                 135                 140

Ile Asn Asp Trp Tyr Asp Arg Asp Ile Asp Ala Ile Asn Glu Pro Tyr
145                 150                 155                 160

Arg Pro Ile Pro Ser Gly Ala Ile Ser Glu Asn Glu Val Ile Thr Gln
                165                 170                 175

Ile Trp Val Leu Leu Leu Gly Gly Leu Gly Leu Gly Ala Leu Leu Asp
            180                 185                 190

Ile Trp Ala Gly His Asp Phe Pro Ile Ile Phe Tyr Leu Ala Leu Gly
        195                 200                 205

Gly Ser Leu Leu Ser Tyr Ile Tyr Ser Ala Pro Pro Leu Lys Leu Lys
    210                 215                 220

Gln Asn Gly Trp Ile Gly Asn Phe Ala Leu Gly Ala Ser Tyr Ile Gly
225                 230                 235                 240

Leu Pro Trp Trp Ala Gly Gln Ala Leu Phe Gly Thr Leu Thr Pro Asp
                245                 250                 255
```

-continued

```
Ile Val Val Leu Thr Cys Leu Tyr Ser Ile Ala Gly Leu Gly Ile Ala
            260                 265                 270

Ile Val Asn Asp Phe Lys Ser Ile Glu Gly Asp Arg Thr Leu Gly Leu
        275                 280                 285

Gln Ser Leu Pro Val Ala Phe Gly Met Glu Thr Ala Lys Trp Ile Cys
    290                 295                 300

Val Gly Ala Ile Asp Ile Thr Gln Leu Ser Val Ala Ala Tyr Leu Leu
305                 310                 315                 320

Ser Thr Gly Lys Leu Tyr Tyr Ala Leu Ala Leu Leu Gly Leu Thr Ile
                325                 330                 335

Pro Gln Val Ile Leu Gln Phe Gln Tyr Phe Leu Lys Asp Pro Val Lys
            340                 345                 350

Tyr Asp Val Lys Tyr Gln Ala Ser Ala Gln Pro Phe Phe Val Phe Gly
        355                 360                 365

Leu Leu Val Thr Ala Leu Ala Thr Ser His
    370                 375


<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 6, 9, 12, 15
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 20

tayrtngtng gnhtnaayca                                                  20


<210> SEQ ID NO 21
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: 2, 5
<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 21

Tyr Xaa Val Gly Xaa Asn Gln
 1               5


<210> SEQ ID NO 22
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 9, 12
<223> OTHER INFORMATION: n = A,T,C or G

<400> SEQUENCE: 22

gcrtaraana rnttccadat raa                                              23


<210> SEQ ID NO 23
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<220> FEATURE:
<223> OTHER INFORMATION: translation of SEQ ID NO:22
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: 4, 5
<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 23

Phe Ile Trp Xaa Xaa Phe Tyr Ala
1 5

<210> SEQ ID NO 24
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 24

aaattaaccc tcactaaagg g 21

<210> SEQ ID NO 25
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 25

atacatgatg cagcgaggag c 21

<210> SEQ ID NO 26
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 26

ctctagaact agtggatccc 20

<210> SEQ ID NO 27
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 27

gtattcctat gctaaagctc 20

<210> SEQ ID NO 28
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 28

gaattttcag tagcaactgg 20

<210> SEQ ID NO 29
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

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<400> SEQUENCE: 29

gtaaaacgac ggccagt 17

<210> SEQ ID NO 30
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 30

ctcctcgctg catcatgtat c 21

<210> SEQ ID NO 31
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 31

gtaatacgac tcactatagg gc 22

<210> SEQ ID NO 32
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 32

ttccatggcg aggatgcaag ccgtcacgg 29

<210> SEQ ID NO 33
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 33

ttgaattcac acatctgctg gcccttgtac 30

<210> SEQ ID NO 34
<211> LENGTH: 655
<212> TYPE: DNA
<213> ORGANISM: Triticum aestivum

<400> SEQUENCE: 34

gcacgaggca acattgttca tgtgttgctt ctctgccgtc atagctctat tcaaggatat 60

tcctgatgtt gatggagacc gagattttgg catccaatcc ttgagtgtga gattggggcc 120

acaaagagtg tatcagctct gcataagcat actgttaaca gcctatgggg ctgccactgt 180

agtaggagct tcatccacac acctacttca aaagatcatc actgtgtctg gccatggcct 240

gcttgctgtg acactttggc agagagcgcg gcaccttgag gttgaaaacc aagcgcgtgt 300

cacatcattt tacatgttca tttggaaggt aactaattaa gttgctcgca tatattgtgc 360

attctctaag ccattaaact ttggctatat atgcctaatg attatttgca cttattgtgt 420

cactttcatg cagctattct atgcagagta tttccttata ccgtttgtgc agtaaaattt 480

gtacaagggc cagcagatgt gaactatata tacatgtaaa acaaattata ttactgatga 540

-continued

taccctatcc aatgcttgga ttttccttgt actgtgttat ctgtaattcc atgatctaga 600

gaaagaggca aatgttgggt gtgtaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaa 655

<210> SEQ ID NO 35
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 35

gccaagctcg gaattaaccc tca 23

<210> SEQ ID NO 36
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 36

cacagtacaa ggaaaatcca agca 24

<210> SEQ ID NO 37
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 37

gccgctctag aactagtgga tcccc 25

<210> SEQ ID NO 38
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 38

tccaagcatt ggatagggta tca 23

<210> SEQ ID NO 39
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 39

ttggaattcg tggccgcccg gcgaggatgc 30

<210> SEQ ID NO 40
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 40

ttggtacctc acatctgctg gcccttgtac 30

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<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: 12
<223> OTHER INFORMATION: n = A,T,C or G
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 41

athgayaarr tnaayaarcc 20

<210> SEQ ID NO 42
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Translation of SEQ ID NO:41
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: 4
<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 42

Ile Asp Lys Xaa Asn Lys Pro
1 5

<210> SEQ ID NO 43
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 43

ggaagtgcat actctgttga tg 22

<210> SEQ ID NO 44
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 44

cttgtatact atttgtaaga gc 22

<210> SEQ ID NO 45
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 45

aacagctatg accatg 16

<210> SEQ ID NO 46
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 46

ataattgctc atgtgcatgg tc 22

-continued

<210> SEQ ID NO 47
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 47

catgtaaatg atgtgatcca c 21

<210> SEQ ID NO 48
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 48

ttgcggccgc agacgatgca agcctcatcg g 31

<210> SEQ ID NO 49
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 49

ttgcggccgc cttgcccttg tgtatatagt gc 32

<210> SEQ ID NO 50
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 50

ttcccgggag acgatgcaag cctcatcg 28

<210> SEQ ID NO 51
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 51

ttggtaccgt gtatatagtg ctcactgcac 30

<210> SEQ ID NO 52
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 52

ttgcggccgc aggatgcaag ccgtcacggc ggcagccg 38

<210> SEQ ID NO 53
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

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<400> SEQUENCE: 53

ttgcggccgc ttcacatctg ctggcccttg tac 33

<210> SEQ ID NO 54
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 54

atttaattaa gccggcgagg atgcaagccg tc 32

<210> SEQ ID NO 55
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 55

tattaattaa ttcacatctg ctggcccttg tac 33

<210> SEQ ID NO 56
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 56

Phe Cys Arg Pro His Thr Ile Phe Gly Thr Ile Ile Gly Ile Thr Ser
1 5 10 15

Val Ser

<210> SEQ ID NO 57
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 57

Leu Cys Met Asn Ile Tyr Val Val Gly Leu Asn
1 5 10

<210> SEQ ID NO 58
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 58

Leu Gly Ser Ala Tyr
1 5

<210> SEQ ID NO 59
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

-continued

<400> SEQUENCE: 59

Leu Arg Trp Lys Arg
1 5

<210> SEQ ID NO 60
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 60

Leu Ala Ala Ser Cys Ile Leu Phe Val Arg
1 5 10

<210> SEQ ID NO 61
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 61

Leu Ala Phe Phe Ala His Met Gln Gln His Val Leu Lys Arg Pro Leu
1 5 10 15

Ala

<210> SEQ ID NO 62
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 62

Val Phe Ala Thr Leu Phe Met Cys Cys Phe
1 5 10

<210> SEQ ID NO 63
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 63

Val Ile Ala Leu Phe Lys Asp Ile Pro Asp
1 5 10

<210> SEQ ID NO 64
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 64

Gly His Gly Leu Leu Ala
1 5

<210> SEQ ID NO 65
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

-continued

<400> SEQUENCE: 65

Leu Trp Gln Arg Ala
1 5

<210> SEQ ID NO 66
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: conserved motif

<400> SEQUENCE: 66

Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu Ile
1 5 10 15

Pro Phe

<210> SEQ ID NO 67
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 67

cgtaaaatcg tccaggctct ttc 23

<210> SEQ ID NO 68
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 68

gcctcgagaa atccccatat agc 23

What is claimed is:

1. An isolated polynucleotide comprising:

a) a nucleotide sequence encoding a polypeptide that increases tocotrienol content, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2 have at least 70% sequence identity, or

b) a nucleotide sequence that is complementary to the nucleotide sequence of a), wherein the complement and the nucleotide sequence contain the same number of nucleotides and are 100% complementary.

2. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2 have at least 75% identity.

3. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2 have at least 80% identity.

4. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2 have at least 85% identity.

5. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2 have at least 90% identity.

6. The polynucleotide of claim 1, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO: 2, have at least 95% identity.

7. An expression cassette comprising the polynucleotide of claim 1, wherein the polynucleotide is operably linked to at least one regulatory sequence.

8. The expression cassette of claim 7, wherein the regulatory sequence is a promoter.

9. A non-human host cell transformed with the expression cassette of claim 7.

10. The host cell of claim 9, wherein the host cell is a plant cell.

11. A transformed plant comprising In its genome at least one stably incorporated polynucleotide of claim 1 operably linked to a promoter that drives expression in a plant cell.

12. The plant of claim 11, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred and developmentally regulated promoters.

13. The plant of claim 11, wherein the plant is a monocot.

14. The plant of claim 13, wherein the monocot is selected from the group consisting of maize, wheat, rice, sorghum, barley, millet and rye.

15. The plant of claim 11, wherein the plant is a dicot.

16. The plant of claim 15, wherein the dicot is selected from the group consisting of soybean, *Brassica* sp., alfalfa, safflower, sunflower, cotton, peanut and potato.

17. Transformed seed of the plant of claim 11.

18. The seed of claim 17 wherein the tocotrienols are increased to between 19 and 461 ppm.

19. The seed of claim 18 wherein the tocotrienols are increased to between 100 and 400 ppm.

20. The seed of claim 19 wherein the tocotrienols are increased to between 250 and 350 ppm.

21. An isolated nucleotide molecule comprising a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO: 1;

b) a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 2;

c) a nucleotide sequence having at least 80% sequence identity to the nucleotide sequence set forth in a), wherein the nucleotide sequence encodes a polypeptide that increases tocotrienol content; and

d) a nucleotide sequence that is complementary to the nucleotide sequence of a), b), or c).

22. An expression cassette comprising the nucleotide molecule of claim 21, wherein the nucleotide sequence is operably linked to at least one regulatory sequence.

23. The expression cassette of claim 22, wherein the regulatory sequence is a promoter.

24. A non-human host cell transformed with the expression cassette of claim 22.

25. The host cell of claim 24, wherein the host cell is a plant cell.

26. A transformed plant comprising in its genome at least one stably incorporated nucleotide sequence of claim 21 operably linked to a promoter that drives expression in a plant cell.

27. The plant of claim 26, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred, and developmentally regulated promoters.

28. The plant of claim 26, wherein the plant is a monocot.

29. The plant of claim 28, wherein the monocot is selected from the group consisting of maize, wheat, rice, sorghum, barley, millet and rye.

30. The plant of claim 26, wherein the plant is a dicot.

31. The plant of claim 30, wherein the dicot is selected from the group consisting of soybean, *Brassica* sp., alfalfa, safflower, sunflower, cotton, peanut and potato.

32. Transformed seed of the plant of claim 26.

33. A method for increasing tocotrienol content as compared to wild-type, in a plant or part thereof, the method comprising transforming the plant with a nucleotide construct comprising a nucleotide sequence of claim 1.

34. The method of claim 33, wherein the part is seed or grain.

35. The method of claim 33, wherein the nucleotide construct further comprises an operably linked promoter that drives expression in a plant cell.

36. The method of claim 35, wherein the promoter is selected from the group consisting of seed-preferred, constitutive, chemically regulated, tissue-preferred, and developmentally regulated promoters.

37. The method of claim 33 wherein antioxidant activity in the plant or plant part is increased.

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